

2 September 1982

Too-easy-to-lose Wall Street blues

Stocks have been rallying and rates of both interest and inflation falling. Is the beginning of the end of the recession therefore in sight or is there more misery to come?

Does the recent excitement on Wall Street and the rallies that have followed on other stock exchanges, mean that the long recession is coming to an end? It is natural that people should now be asking this hopeful question just as it is natural that a man lost in a desert should mistake refraction of light in the hot air near the ground for a pool of water. For if the people who make their livings by buying and selling stocks in commercial corporations now think it worthwhile to pay more for the commodities in which they trade, does that not imply that the outlook for US industry has improved? And does it not follow that industrial economies elsewhere will soon be on the mend? Unfortunately these conclusions, welcome though they would be, are unwarrantable. This is why.

Last week's rally on the stock exchanges followed from two significant developments in the United States — a modest but steady decline in the prevailing high interest rates and the government's success in persuading Congress, on the eve of its summer holidays, to enact a package of tax increases that will bring in an extra \$98,300 million over the next three years. If the financial community had waited a few days, it would no doubt have been further cheered to know that the annual rate of inflation in the United States has now fallen to 6.7 per cent. While each of these developments is a sign of progress in the right direction, neither of them implies that the end of the recession is at hand.

The decline of interest rates to below 14 per cent (when banks lend to each other or when people lend to the government) may save many industrial corporations from bankruptcy, but there is as yet no sign that corporations will seize this opportunity to invest in new plants and equipment. Why should they when they have to pay more than 17 per cent for the funds they borrow from the money market and the bank? A decline of interest rates is rather a sign that industry has virtually dropped out of the competition for people's savings, leaving the government as the only borrower of significance. This development is therefore more a sign that the recession has begun to bite than the promise that it is coming to an end.

On present form this will remain the case for some time to come. The package of tax increases half-heartedly proposed by the government and reluctantly swallowed by Congress will not do much to help. The government's deficit for the present financial year (which ends on 30 September) will be untouched at \$110,000 million or more. The tax increases now enacted will be effective only after 1 October, and are smaller than last year's tax reductions, also then coming into effect, and will not in themselves prevent the government's deficit actually increasing in future years. The hopeful sign and the only reason why Wall Street is excited is merely that the Reagan Administration appears at last to have recognized that it cannot run deficits on this scale without also running the economy into the ground. That a Republican administration should so lightly have thrown to the wind the traditional (and old-fashioned) Republican preoccupation with balanced budgets, remains a puzzle, while Mr Reagan's support for the proposed constitutional amendment that would compel future governments to balance their books is about as easily understood as a burglar's espousal of the calls of law and order.

The chances now are high that when the November elections are safely past, the President will make another attempt to behave as he says he should and will ask Congress to agree to a more substantial reduction of the social expenditure to which it has committed him. That will be a more bloody battle than that now won. The outcome cannot be foretold, but success will mean further misery for the growing army of the unemployed, now 9.7 per cent of the working population.

Meanwhile, there is precious little evidence that the transformation of the US industry has gone far enough to provide a foundation for future prosperity. As in most of Western Europe the privations of the recession have not yet made conventional manufacturing industry competitive with similar industries elsewhere, principally in Japan. Indeed, in the manufacture of steel and motor cars for example, the chief preoccupation is still to keep some semblance of these industries alive when there is no reason to suppose that they have an important place in the world. The time to celebrate the impending end of the recession will be when tangible signs of new industries emerging and of improved productivity in conventional industries are plain for all to see.

A very different game

Can French success in boosting nuclear and space technology be repeated in electronics?

A few weeks ago, the French government announced that it was to invest FF140,000 million (£12,000 million) in the French electronics industry over the next three to five years — an apparently vast sum by all measures (see *Nature* 5 August, p.504). But now the dust is beginning to settle, it seems the amount of new government cash may not be so great. Some FF 90,000 million of the total was already due to be invested by the industry itself; and of the remaining FF50,000 million promised by the government (none of which has yet materialized) most can be accounted for by money already budgeted by the industry to be raised from the banks and other sources now nationalized.

So what is the great French electronics plan really all about? Central planning, it seems, despite President Mitterrand's insistence that the leadership of the nationalized industries would be free to manage their industries as they wished. What the President failed to emphasize was that the leadership would be chosen by the government.

So, for example, when the president of the newly-nationalized giant chemicals corporation, Rhône-Poulenc, felt obliged to resign recently (in protest against the "inconsistency" of government policy for his industry), the matter was dismissed by research and industry minister Jean-Pierre Chevènement in revealing terms. The state was in need of "good servants", he said. Jean Gandois, the resigning president, replied that he had found himself to be a "hostage" of the government.

With the leadership of much of industry in its pocket, therefore, the government must now manage industry — something that the present British government would consider to be a logical impossibility. In France, the division between government and industry is not so sharp; the leaders of both



sectors tend to come through the same somewhat military system of education — the *grandes écoles* — and interchange and interaction between civil service and industry is traditionally much stronger in France than in Britain.

Thus the change under Mitterrand is not so much a change of principle as of degree. There is, however, in this socialist government, an increased tendency to make appointments on political and ideological grounds, and voices in the electronics industry in particular are beginning to question the experience and suitability of some of their new leaders.

Moreover, the government has selected electronics to be the centrepiece of its five-year plan for the regeneration of French industry, putting the field on a par with nuclear power and space technology — the other areas very successfully championed by past French governments. But electronics is a very different game. In nuclear power, and in space, there is essentially one customer — the government — at least in the first decade or so while the business is being established. Here, a simple military regimentation of the industry towards well-defined goals will work, as all levers are in the government's hands. But in consumer electronics, for example, there are millions of customers, and there must be flexibility in response to changes in demand and competition. Even the products may change radically. It is doubtful if even the French government machine will be supple enough to deal with this by central planning, however complex the plan.

It may be, therefore, that the great government effort on electronics will work only in those areas where electronics is most like nuclear power and space, in having small, well-defined markets with few customers. This would mean that France should be watched now not for its calculators or video recorders, nor for its consumer products, but for what is called "professional electronics" — defence equipment, where there is a large French and foreign market, electronics for broadcasting and so on.

However, there is one clear thing that a great centralized plan for electronics could do. The importance of components (chips) and of software is increasing in all parts of the industry as one influential (and sceptical) director in the French electronics industry, M. Pierre Aigrain, points out. If the government can put these crucial parts of the French electronics house in order, and bring them to bear on the rest of the industry, whatever can be done by central control will have been done. Other, that is, than providing some real (rather than promised) investment.

Father William's jaw

The cost of caring for old people in the United States will grow dramatically if nothing is done.

Last week's \$98,300 million tax bill, passed by the US Congress with such fanfare, held portents of future problems for old people in the United States, for it showed the tremendous pressures that have mounted on the cost of caring for them. And while scientists like to think that the orderly world of their laboratories is far removed from political fights over Medicare and Medicaid, the research priorities of biomedical scientists in the next few years could have an impact on future costs of caring for the elderly, not to mention an impact on their well-being. US biomedical research and social policy, then, are linked.

The new law, for example, will reduce the chief health programme for the elderly, Medicare, which now costs \$53,000 million, by \$13,300 million over the next three years and increase the costs paid directly by old people by, for example, making elderly hospital patients bear some of the costs of radiology and pathology.

The move to reduce costs collides head-on with the increasing number of old people in the US population, whose government-supported health care will have to be paid from the taxes of a relatively smaller group of younger, working people (*Nature* 26 August, p.779). The resulting battle will make this year's skirmishes between the "gray lobby" seeking to retain federal

benefits, and Reagan-inspired budget cutters, look almost trivial.

Matters are not helped by the fact that the present system of Medicare and Medicaid encourages doctors to treat hospitalized patients with acute problems while most custodial care, long term care and outpatient care, is not covered.

The system reinforces what Robert N. Butler, the outgoing director of the National Institute on Aging, calls "Peter Pan Medicine": a young or middle-aged person with an acute problem but good chances of recovery is a subject of interest and is given the maximum care at no extra cost, while an older person, whose health problems are limited to outpatient visits, is not allowed to charge to the government the cost of medication and, if in hospital, is considered a less interesting case and so gets relatively little attention.

Even worse off are those in nursing homes, for long the stepchildren of the health care system. Federal insurance does not extend to the costs of nursing homes and Medicare pays only for those who have entered a home after an acute illness in hospital, and then only for 100 days. Meanwhile there is no evidence that nursing homes are any good, having been divorced from the mainstream of research, teaching hospitals and medical practice for so long. There are no teaching nursing homes, although the National Institute on Aging plans to try some, and there is currently only one registered nurse for every 68 residents in the country's 60,000 nursing homes. Many do not even have a regular doctor available.

Demographics will only make this worse. There were 1.3 million people in nursing homes in 1978; there will be 2.1 million in the year 2003. In the present political climate it is impossible to believe that federal benefits will be extended to include long-term care. But could not the need for long-term care be reduced by a better understanding of its medical causes? Why are people in nursing homes to begin with? Could science enable nursing homes to go the way of the TB sanatorium?

Most people enter long term care because they are senile, many of them suffering from Alzheimer's disease. Here, at least, recent progress in understanding the disease at the cellular level and its possible association with slow viruses holds out some hope. Another major reason people are sent to nursing homes is urinary incontinence, about which little is known. Next come infectious diseases, notably pneumonia and influenza, but also herpes zoster (shingles), tetanus and even tuberculosis and chickenpox, causing one in four deaths of elderly people. Here, a better understanding of the effects of ageing on the immune system could help.

Finally, there are bone fractures, encouraged by osteoporosis which could perhaps be minimized by better care in middle age and early old age, perhaps through fluoride and calcium supplements, treatments with oestrogen, progesterone, and vitamin D, and even exercise. Butler goes farther to say that biomarkers, "clocks" to detect how far organ and bodily functions have aged, may make it possible to run short clinical trials, obviating the need to wait many years for a result. This could make possible more direct study of the ageing process, and whether factors such as early diet are linked to old-age symptoms.

These are long-term goals but not far fetched ones considering the high potential social payoff. The "gray lobby", caught in yearly battles, seems unlikely to make a high priority of broad-based biomedical research, with its ifs and buts and necessarily long-term payoffs. It is the research community who hold the keys to the elderly's best hopes 20 years hence and who should take the initiative.

Only the scientists can perhaps ensure that we approach the ideal of a healthy older person, who is not senile, whose bones are not too frail, who is not humiliated by incontinence and who does not die of diseases a child would shake off.

The elderly might even come to resemble Lewis Carroll's Old Father William whose muscular jaws and hearty eating were attributed to the arguing he did as a youngster. Old Father William could stand on his head, somersault backwards, eat a goose, bones and beak, and kick his son downstairs. One thinks of him as a shrewd old fellow, who surely did not cost the state any money.

Pipeline embargo in new jeopardy

Soviet Union's technology could adapt

President Reagan's ploy to stop or delay the supply of Siberian natural gas to Western Europe may all be in vain — because of two simple technical options open to the Soviets.

Reagan's tactics have been to urge European steel manufacturers — particularly West Germany — to stop supplies of the large-diameter (1.42m) steel pipe needed for the line; and to forbid General Electric and other American suppliers from shipping key parts (for example turbine blades) to Western manufacturers making the gas turbines and compressors needed to pump the gas.

But the Soviet Union is experimenting with increasing the pressure of gas in the line from its nominal 75 atmospheres to 100 or even 120 atmospheres. At 100 atmospheres, the pipeline could be built with only two parallel pipes (the current design has three) and yet carry the same net flow of gas (40,000 million standard m³ a year). At 120 atmospheres, only one pipe would be needed, according to official reports in the newspaper *Pravda*. This would reduce the steel requirements for the line to within the capacity of Soviet industry (although the higher pressure pipes would have to be reinforced), and it would also drastically reduce the cost.

Another neglected fact is that the design capacity of the line is in excess of the contracts for gas so far signed in Europe. Since the power required to pump gas down a pipeline rises more than linearly with the rate of gas pumped, it would be possible for the Soviets to pump the contracted gas with many fewer than the 125 gas turbines of 25 MW currently on order in Europe. The 125 turbines are only necessary to pump the full design flow rate.

Exactly how many turbines the Soviet Union would need to be in place by 1984 — when the line is supposed to come on stream — depends on the detailed characteristics of the line. However, the plan is to supply just 15,000 million m³ at that date — under 40 per cent of capacity. It was never envisaged that the line would be pumping its full capacity before 1987.

In fact, according to British pipeline engineers, it would be presumptuous to assume that the Soviets would need as much as 15/40 of design pumping power by 1984, that is 47 or so 25-MW turbine and compressor sets.

It happens that there are parts available in Europe to supply 23 such sets to the Soviet Union, six at John Brown in

Scotland and the rest in France and Italy. That would leave the Soviet Union with at most 24 sets to supply from its own industry in Leningrad, as opposed to the 102 apparent from the design. According to British turbine manufacturers, the General Electric turbines are not particularly sophisticated technology — they are based on 1950s steam turbine design. The obstacle to their manufacture in the Soviet Union is primarily a matter of industrial capacity.

At present, the Soviet Union has no such capacity for 25-MW sets, but West European engineers are convinced that a working prototype is now under test in Leningrad. According to Soviet sources this prototype is "even more efficient" than the General Electric sets it is designed to replace. Whether the Leningrad works could supply 24 of these sets in working order by 1984 is open to question, but the target is a good deal more realistic than the 102 that the Reagan embargo apparently implied.

Another option open to the Soviet Union is to divert 10-MW set production, for which it has capacity, from the six national pipelines under construction. And

in Europe, the French company Alsthom-Atlantique has the capacity to build complete turbines (albeit under licence from General Electric, and so in defiance of the Reagan embargo and so in risk of penalties in America).

Thus it seems entirely conceivable that one way or another the Soviet Union will meet its 1984 deadline — and that it could develop the capacity to bring the line up to full flow by 1987, as Soviet sources continually affirm.

The pipeline design requires three 4,500 km strings to bring the gas from Siberia to the Western border of the Soviet Union. So far, 2,700 km of pipe have been delivered to the pipeline builders, according to the Soviet news agency Tass. Some 500 km of pipe have been welded into a single line, says Tass, and by mid-August 250 km had been laid in place.

Pipe laying equipment which was to have been supplied by the American Caterpillar company is also embargoed, but a new Soviet-designed pipe-layer is now under test, says Tass. The Japanese company Komatsu is also substituting for Caterpillar equipment, according to the news agency.

Robert Walgate and Vera Rich

EPA holds out against lead

Washington

The US Environmental Protection Agency (EPA) stuck to its guns last week and issued a regulation tightening limits on lead in gasoline, despite pressure from the Office of Management and Budget (OMB) to back off.

The new rule, to take effect on 1 November, limits the lead content of leaded gasoline to 1.1 grammes per gallon. EPA estimates that after eight years, airborne lead concentrations will be 31 per cent lower than they would be without the new rule.

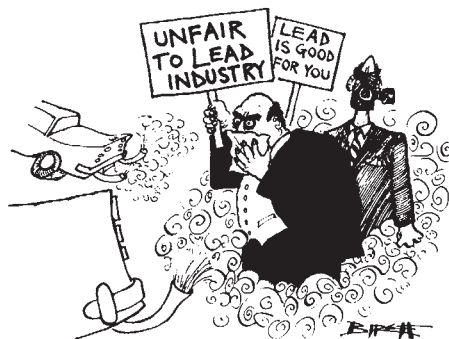
Under current regulations, refiners are allowed to average the lead content of all their gasoline, both leaded and unleaded; that pool average must not exceed 0.5 grammes per gallon. But as a result, refiners have been increasing the lead content of leaded fuel (which cannot be used in new cars with catalytic converters) as demand for it drops off. Adding lead is the cheapest way of increasing octane ratings, and EPA has found refiners using as much as 2 grammes of lead per gallon.

Earlier this year, EPA had proposed relaxing the lead regulations. This was done at the bidding of Vice President George Bush's task force on regulation. But in the face of a storm of protest — and convincing evidence that reductions to date in airborne lead have brought about real reductions in blood-levels — EPA went back to the drawing board.

By the beginning of August, EPA had

completed its about face, and in an apparent attempt to out-flank OMB, leaked its new, tougher proposal to the *New York Times*. OMB nonetheless responded by requesting EPA to reconsider the proposed 1.1 gramme limit but EPA has held its ground.

OMB did get its way, however, over the issue of the so-called small refiners.



Current regulations grant an exemption for refiners who produce less than 50,000 barrels per day; they are allowed to add from 0.8 to 2.65 grammes of lead per gallon, according to their scale of production. The new rules will narrow the exemption, reserving the "small refiner" designation for producers of less than 10,000 barrels a day who were in business before October 1976. According to EPA, this will leave only 74 companies in this category, about half the current number. They will be allowed to add up to 2.5

grammes per gallon. The losers will be the "blenders", companies that jumped into the business in the past few years to take advantage of the small-refiner loophole. They buy cheap gasoline, add lead to boost the octane, then resell it. That practice should be largely halted by the new rules.

Environmental groups are generally pleased with the new rules. The lead industry, predictably, is not. In a letter to the *New York Times* last week, Dr Jerome Cole, vice-president of the International Lead Zinc Research Organization argued that the new regulations will cost the public "millions of barrels of crude oil that lead in gasoline saves while adding billions of dollars to the US balance of payments deficit."

Stephen Budiansky

Affirmative action employer

The launch of the Soyuz-T, with a three person crew including female cosmonaut Svetlana Savitskaya, coincided neatly with the closing of Unispace-82 in Vienna and upstaged the US contribution to equal opportunities in space, the visit to the conference of Dr Anna Fisher, astronaut in training. Miss Savitskaya's visit to Salyut-7, however, should not be viewed simply as a publicity gimmick, nor an attempt to scoop the launch of Dr Sally Ride aboard the Shuttle next spring. The fact that there were female candidates training at the Gagarin space centre was announced some weeks ago. It would seem that, as far as the space planners were concerned, the launching of a woman was the next routine step.

Soviet space policy is strongly committed to the construction of large space stations,



Savitskaya and crew-mates

aboard which women would serve as scientists. ("And, of course, stewardesses", Andrian Nikolaev, the husband of the first Soviet woman cosmonaut Valentina Tereshkova, once added.) Studies of the effect of spaceflight on the female organism are an obvious prerequisite of such a programme. Yet, since Tereshkova's solo flight in 1963, no woman has been placed in orbit. The reason appears to be partly one of what a Soviet space official delicately called "the amenities". Moreover, the 1961 Soyuz-11 disaster, in which three cosmonauts died due to loss of cabin pressure during re-entry, led to a change in procedure; cosmonauts were to wear spacesuits during the re-entry, which meant that crew size had to be reduced from three to two. It was the introduction of the roomier Soyuz-T transport craft and Salyut-7, that made it possible for the multi-crew spacecraft to have a female visitor.

Israeli science politics

Physicist made Science Minister

Rehovot

Professor Yuval Ne'eman, a well known theoretical physicist and former president of Tel Aviv University has become Israel's first Minister of Science, just five years after turning down the post because he preferred to stay out of politics. Since then, though, Ne'eman has become a fully-fledged politician and now represents the nationalist Tehiya Party in the Knesset. When Tehiya joined the Begin-led coalition government, Ne'eman accepted the position of Minister of Science and Development.

Not all of Ne'eman's academic colleagues are enthusiastic about the notion of a ministry with overall responsibility for science. For one thing, they fear that it might mean an undesirable degree of government control. Ne'eman discounts such fears and claims that there are overwhelming benefits in having science represented at cabinet level. Other ministries already have their own chief scientists and research budgets and Ne'eman sees one of his chief tasks as introducing strong central coordination over these separate activities.

Professor Ne'eman is pleased with what has been achieved by Israeli scientists and technologists, but looks forward to a "quantum leap" in these achievements, in particular supporting the idea of creating "science cities". And he has set a target of \$5,000 million dollars a year for the annual income from exports based on local research — the current level being only \$1,000 million.

Although Ne'eman is clearly putting the emphasis on applied research, he says he will also be fighting to see that pure research gets the funds it deserves. He is particularly interested in creating more national experimental facilities like the Weizmann Institute's nuclear accelerator and the 40-inch telescope at Tel Aviv University. He also hopes to explore the possibility of Israel's becoming involved in further multi-national research bodies. Already Israel is a member of the European Molecular Biology Organization, and other candidates are the European Southern Observatory and the European Space Agency.

Only in the past 15 years, says Ne'eman, has advanced science and technology begun to have a serious impact on Israeli industry. Ne'eman himself can claim much of the credit — during the sixties he was amongst those who persuaded the government to back skill-intensive science-based industry at the expense of the labour-intensive textile industry and in the mid-seventies, as Chief Scientist in the Ministry of Defence, he had a significant impact on the country's military science.

Some Israeli scientists are sceptical about one of Ne'eman's pet projects, how-

ever. He is committed to the plan to build a canal from the Mediterranean to the Dead Sea, which among other things will provide hydroelectric power by utilizing water from the hills around the Dead Sea. Some question the value of spending an estimated \$1,000 million on a project that would only provide a few per cent of Israel's energy requirements. Ne'eman, for long a moving spirit behind the plan, maintains that the energy would be available at crucial times and that the canal



Ne'eman takes science to the cabinet

would provide much-needed cooling water for additional thermal power stations along the route.

Looking forward to his new task, the new minister says he will do his best "not to disconnect" from "real science". "I was serving as a military attaché with the Israeli Embassy in London," he recalls, "when I worked with Murray Gell-Mann on 'The Eightfold Way', the theory that led to the prediction of quarks. And if I was able to combine the purchasing of submarines with the charting of elementary particles then, I don't see why I can't maintain the same duality now." **Nechemia Meyers**

US degrees

Doctoral decline

Washington

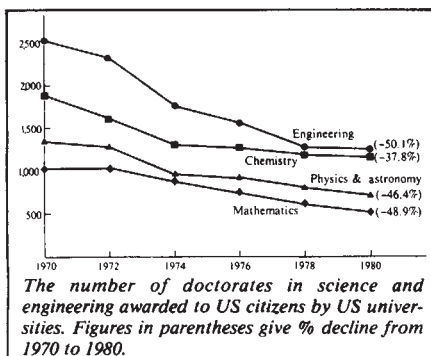
The number of US citizens who received doctorates in the "hard" science fields in the United States declined steadily during the 1970s (see chart). Some see in this trend a dangerous drift away from basic research as a career priority for young US scientists. David A. Shirley, director of the Lawrence Berkeley Laboratory, considers the figures "poignant" evidence of that US society is steering its young people away from basic science.

Another explanation is the changing environment in US university science departments, and the steady upward trend in salaries offered by industry to graduates who have made the initial four-year invest-

ment in a bachelor's degree in the hard sciences or engineering.

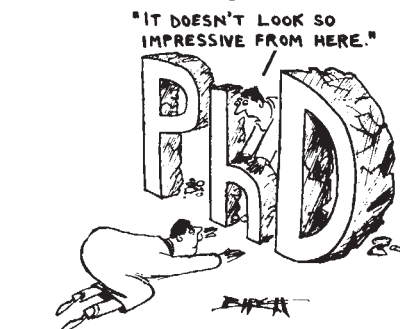
When US university science departments were expanding and funding was ample, a career in science was seen as the direct pursuit of a PhD and a research career on a university faculty. Young people were encouraged to consider this the most promising route — it being also the one their faculty advisers had usually followed.

But now there are few positions available to a young PhD in university departments



and laboratories, and still fewer with prospects of a permanent university job with tenure. Furthermore, industry pay is better. One recent survey found that industry offers geologists with a master's degree much the same salary (up to \$24,000) as they would command with a PhD as an assistant professor. So increasingly, young US researchers may not see academia as a promising or even stable place to be.

So, should they bother to get a PhD before entering industry? In June 1982, new PhDs in mathematics were offered an average of \$30,000 a year by industry, whereas new bachelors' degree holders in the field were offered, on average, \$9,000 less. Chemical engineers with a PhD were offered on average \$36,000 and again those with bachelors' degrees were offered



\$9,000 less. Since a doctorate takes seven years to earn, and the candidate earns little or no money during that time and must pay for tuition, the \$1,300 reward per year of study for the degree might seem insufficient.

So economics may be reshaping student attitudes away from the traditional career of a PhD and a university post. Of the situation, they may even be thinking, as one manpower specialist said, "why can't I do good work in industry, too?"

Deborah Shapley

Artificial intelligence

Industry beckons students

Pittsburgh

When 1,500 of the hard core among computer adepts converged upon the University of Pittsburgh campus for the National Conference on Artificial Intelligence (AI) recently, the usual talk of knowledge representation, the meaning of contradiction and many dozen other steps on the long road to a computer with human-like reasoning and perception, was supplemented by talk of entrepreneurship and commercialization. This is something new for the infant field of AI, and it has the field's old guard worried.

The change was apparent in both the exhibitors at the meeting and the audience; Dr Roger Schank of Yale University, who is also the founder of Cognitive Systems Inc, a prototype of small entrepreneurial ventures in AI, noted that ten years ago not a single venture capitalist was to be seen at AI meetings. This year, Cognitive Systems was one of several firms showing off specialized information systems for applications such as petroleum exploration, molecular genetics research and financial analysis.

Leading the charge against the new commercialism was Professor Marvin Minsky of the Massachusetts Institute of Technology, the "dean" of AI. "What I don't like about the problems most people work on is that there's too much 'do-goodism' in them," he said. Applications, such as information systems to aid in medical diagnosis or petroleum exploration draw talented people away from work that will do greater good in the long run. Minsky warned that most of the basic research in AI is done by doctoral students who then

go to work for a company, applying the AI expertise of several years ago on some very specific problem, such as signal processing (or, as Schank put it, "expert systems on paint thinners"). In so doing, Minsky said, "they rob the world of their intelligence".

The lure of industry is also attracting established university researchers in AI, just as it has in other engineering fields. A substantial number of people moving into industry are at the middle level, according to Professor Allen Newell of Carnegie-Mellon University. Newell said these are the researchers who would otherwise just be taking over their own research programmes at universities, and "we're really going to feel that loss".

According to Newell, universities should be made more competitive, both in salaries and research facilities. The average university researcher, he said, receives only one-half to two-thirds of the research support that an industry researcher receives.

To be sure, what is now happening to AI is no different from what has happened for years to computer science and engineering in general. AI, however, may be much more vulnerable. It is still a very young and speculative field, and thus pressures from industry for quick pay-backs are at once stronger and more acutely felt. The result, said Minsky, is that in the entire field there are less than 100 people who have a "license to kill" in having the freedom to spend five or ten years on a single project.

And Minsky offered a history lesson to those who expect quicker results. "It was 300 years from Galileo to Einstein," he said. "What could those fellows have been doing?"

Stephen Budiansky

University woos Oak Ridge laboratory

Last May, when the Union Carbide Corporation announced it would not renew the contract it has long held with the government to manage the Oak Ridge National Laboratory (ORNL) and three related atomic facilities, most observers expected some other corporation to take over (*Nature* 27 May, p.255).

The bidding has barely begun; indeed the US Department of Energy is only just asking all who are interested to make themselves known. Surprisingly, the local University of Tennessee at Knoxville, looks the most serious contender.

L. Evans Roth, the university's Vice Chancellor for Graduate Education, argues that the ORNL and the university are already intertwined, with ORNL scientists teaching at the university and the university programmes under way at the lab. "We already have a marriage without the contract", Roth says.

There is little doubt that acquiring the highly prestigious multi-disciplinary lab-

oratory would be a feather in the university's cap, and to that end it has a task force studying the shape of a management contract, with separate committees on administration, scientific-technical matters, and faculty liaison. Roth and other officials have toured the other national laboratories to look at other university/laboratory relationships, and Glenn Seaborg, the Nobel Prize winner and former atomic energy chief, has visited Knoxville to proffer advice.

Oak Ridge, on the other hand, may have mixed feelings about a union with the University of Tennessee — a state institution with 45,000 students and little experience in managing a giant research enterprise. Now under fire from many in Washington who are questioning the value of places like ORNL, the laboratory might be better served by a more experienced partner. The final decision may depend on what other suitors emerge in the months ahead.

Deborah Shapley

US medical care

Hospices gain

Washington

What could be a major shift in the pattern of US health care lies nearly hidden in the big tax bill Congress passed on 19 August. This is a feature allowing the federal government to pay for hospice care, that is, care of terminally ill people who have chosen not to have extreme life-prolonging measures and want to "die with dignity". Some 95 per cent of US hospice patients have cancer, and an estimated half of them are over 65.

The advent of what amounts to complete federal insurance for hospice care is sure to swell the ranks of those who chose it. Indeed, because this is a budget cutting, cost-saving year for US domestic programmes, the congressmen voted for it because of claims that it would encourage more people to use hospices, relieving pressure on acute care hospitals.

Until now, the estimated 36,000 people receiving hospice care in the United States have had to pay for many of the costs themselves, or have their families pay. Of the estimated 360,000 deaths from cancer per year in the United States, only 10 per cent are now in hospice programmes. So the potential growth of hospice usage is enormous.

According to figures provided by the Congressional Budget Office that Congress relied on heavily in its decision, this extension of Medicare benefits would cost the programme an additional \$1 million per year in the first two years. Afterwards, however, the numbers of people choosing hospices over hospitals would save \$16 million in fiscal 1985 and \$40 million in fiscal 1986. The National Hospice Organization says the cost of home hospice care is up to \$100 a day, compared with \$600 for full hospital care.

The measure's successful passage, despite opposition from the Administration, the American Medical Association, the American Hospital Association, and the Blue Cross Association, has been eclipsed by the larger fight over the tax bill.

The bill has been a focal point for national discussion of how to narrow the federal deficit and give the business community confidence in a US economic recovery. One House staffer deeply involved in the legislation noted that in normal times, something like the hospice measure would have made a big splash at the time it passed. Instead it has barely been reported, having arrived in the final bill by a strange parliamentary route.

In the house the most active proponent was Leon Panetta (Democrat, California), while Senator John Heinz (Republican, Pennsylvania) is responsible for having the Senate version included in the midst of that body's marathon debate on the overall tax bill.

Deborah Shapely

Bankruptcy for interferon firm

Washington

While Southern Biotech Inc., a Tampa, Florida, biotechnology firm that was forced to file for bankruptcy on 28 May, continues to negotiate with its creditors a plan to become solvent again, the US Food and Drug Administration has approved the company's first interferon product for clinical trials.

Approval came on 16 August for US trials of an interferon preparation of possible benefit to lymphoma patients. Although founded and promoted on the basis of its possible interferon sales, the company had not previously been able to market any in the United States, and a 50,000-million unit inventory of alpha-interferon remains unsold.

To facilitate its product reaching doctors in the Florida area, says Daphne B. Floyd, the company's vice-president for marketing, she has helped start a new organization, the Interferon Foundation of Florida, to help tell cancer patients and doctors about interferon. Southern Biotech has donated enough of the newly approved product to the foundation to treat 20 patients.

The company was formed in 1979 by former physician, John Kilgore, who sought to graft an interferon products business using genetic engineering onto a successful blood plasma business. But various deals, including one with Key Energy Enterprises of Tampa, apparently drained the company of cash. Hopes of an innovative research programme under William E. Stewart II, formerly of Memorial Sloan-Kettering Cancer Center, were dashed when Stewart, who was listed

as director of research at the time of the 18 August 1981 stock offering, was fired in September. The prospectus envisaged 17 doctoral-level scientists and a support staff of 34. At present, the scientific staff numbers 3, says Floyd.

However, the company continues to sell its blood plasmas in the United States, while a subsidiary, Southern Biotech Caribbean, located in Kingston, Jamaica, sells its interferon products locally and in West Germany.

Southern Biotech currently has applications for two other interferon products, one for treating breast carcinomas and the other for herpes, filed with FDA, says Floyd.

Deborah Shapely

Spruce bark beetle

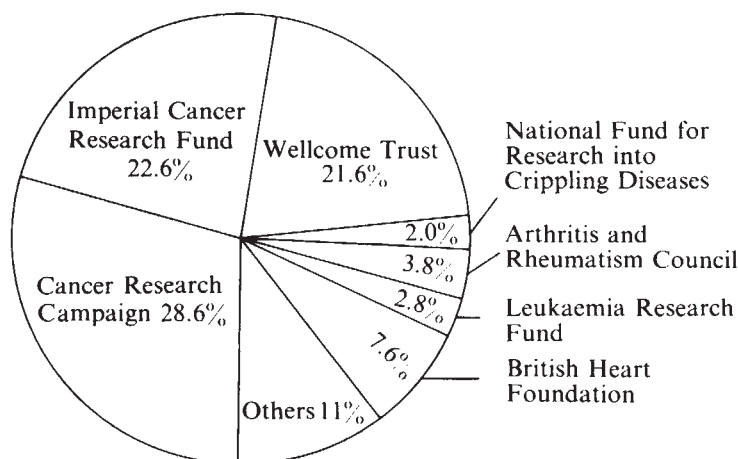
Peril imported

Despite stringent import controls which demand the stripping and spraying of imported conifers, the great spruce bark beetle (*Dendroctonus micans*) has found its way into British woodland.

Forestry Commission entomologists have identified the beetle on a private estate near Ludlow in Shropshire and the commission has implemented a monitoring programme using helicopters and foot patrols to survey the area within a 50 mile radius of Ludlow. Other counties affected are Gloucestershire, Hereford, Powys and Glamorgan. The Forestry Commission is optimistic that the pest will remain confined to this area assuming it has not already spread further afield.

Dendroctonus micans attacks and kills

Per cent distribution of UK charities' £64 million expenditure on medical research



The total annual income of the 35 principal UK medical research charities passed £100 million for the first time last year according to figures collated and just released by the Association of Medical Research Charities. Total expenditure was only £3 million less than the £103 million income but only 64 per cent of the expenditure was on "research" (the Spastics Society spent virtually all of its £18 million on such things as housing and care). The diagram shows the major contributors to research spending; none of the "others" spent more than £1 million. For comparison, the Medical Research Council was granted £102 million by Parliament for 1981-82. And in 1981 the National Society for the Prevention of Cruelty to Children collected £6.7 million and the Royal Society for the Prevention of Cruelty to Animals, £7.8 million.

conifer trees, in particular Sitka and Norway spruce, which account for a large part of British woodlands. It is endemic in Northern Europe where forestry management techniques have already been adapted to its presence. The last serious outbreak was in Holland in 1955 but after a four-year programme the beetle was eradicated.

The female great spruce bark beetle attacks spruce 20–25 years old when the bark is thick enough for her to eat out a small protective chamber where she can lay her eggs. Trees which are suffering stress are particularly vulnerable. The 100–300 eggs, laid in two broods, hatch out and the larvae feed on the edge of the chamber before emerging as adults. In serious cases grubs eat out grooves, causing ring bark.

To contain the attacks, affected trees have to be felled and stripped of their bark which is burned on site or sprayed with insecticide. The Forestry Commission has the power to ensure that owners of woodland, both private and public, take action and destroy infested trees.

It is likely that the beetle arrived in Britain in imported timber that was not properly stripped but, because the life cycle of the beetle varies from one to four years, it is impossible to know how long it has been in Britain. What seems certain is that now *Dendroctonus micans* has crossed the Channel it looks like being a pest we will have to learn to live with.

Jane Wynn

East German scientists

Too many now

Science planners in the German Democratic Republic (GDR) have failed to come up with a coherent policy for the division of labour in science, according to leading East German sociologist, Professor Manfred Loetsch. Speaking on Berlin radio, Professor Loetsch claimed that this bad planning was a major cause of the disparity between spending on science and technology and the rise in productivity in the GDR.

Part of the problem, Professor Loetsch suggested, lies in the pay structure of East German science. In general the GDR has a pay policy based on payment by results. But this principle does not apply in science, where there is a fixed pay scale according to the grade of post.

Incentives, however, are not the only requirement for increased scientific productivity. Science in the GDR, said Professor Loetsch, is greatly hampered by the lack of "intermediate personnel". Higher education was greatly expanded during the last decade, but this expansion has not been matched by a parallel growth in the training of technicians. Indeed, the science planners seem to have neglected the need for such auxiliaries, so that whereas the international average for technical research gives six auxiliaries to one scientist, in the GDR the ratio is 0.8 to one. Frequently, Professor Loetsch complained,

Embarrassing admissions cause trouble

Mathematicians and human-rights activists in the West are preparing a petition to appeal for the release of a Soviet colleague, Valerii Senderov, formerly a teacher in one of the Soviet Union's schools for mathematically gifted children. Senderov was arrested in June and is now in pre-trial detention. He will probably be charged under article 70 (anti-Soviet agitation) or article 190/1 (slandering the state).

From the official Soviet point of view, Senderov is a double offender. In 1978 he was a founder member of SMOT, the unauthorized "Inter-professional Free Trade Union" group that has recently faced increasing official hostility (founder member, Albina Vakoreva arrived in Vienna last week, after being forced to emigrate). In addition, Senderov, together with fellow mathematician Boris Kanevskii, has for the past four years been monitoring admissions to Moscow University's faculty of mechanics and mathematics.

Senderov's group claims to have established discrimination against Jews.

In 1979, the team found that of 48 applicants with no Jewish parents or grandparents, who had between them won 26 prizes in the Moscow and All-Union "Olympiads", 38 were admitted to university on the result of their entrance examinations and a further two on appeal. From a group of 40 having at least one Jewish grandparent, who between them gained 48 prizes in the Olympiads, only four were initially admitted and a further two on appeal. The group's most recent report, signed also by mathematicians Naum Meinan and Grigorii Freiman, shows the same pattern for 1980, 41 non-Jewish candidates admitted out of 49, and two "Jewish" candidates out of fifteen.

The arrest of Senderov on 17 June and of Boris Kanevskii only four days later makes a report on this year's entrance examinations, held in July, very unlikely.

Vera Rich

the lack of auxiliaries is justified on ideological grounds, so that, for example, a scientist who complains that shifting stores to the laboratory is not his job will be castigated as an "elitist". Rather than expand unduly the number of graduates, he said, existing graduates should be better deployed. At present a significant proportion of graduates (estimates range from 12 to 20 per cent) are employed in posts for which a university education is not necessary.

Vera Rich

UK academic posts

Muddy waters

Both the British Home Office and the Committee of Vice-Chancellors of British universities have made moves to try and clear up, once and for all, the confusion that persists over the appointment of overseas candidates to UK academic posts. In a recent letter to the vice-chancellors' committee, the Home Office outlined its interpretation of the immigration laws as applied to university staff. And to help universities abide by that interpretation, the vice-chancellors' committee has appointed a special officer to act as an intermediary in disputed cases.

The confusion seems to have arisen because neither universities nor immigration officials have known how to apply British immigration law to overseas students at British universities who later apply for academic posts. British immigration law is against visitors using their foothold in the country to gain permanent residence. But the Home Office also acknowledges the case for employing foreign academics who could make an outstanding contribution.

Hence, determining who should be allowed to stay has often been a lengthy procedure. Universities have taken exception to officials questioning their judgement on who is suitable for a particular job and candidates have been subjected to undue delay before hearing if their applications have been accepted. In some cases, candidates have even been told to go home to their native countries before applying for a work permit.

The Home Office letter signals no change in the law but tries to set out the criteria to be used in deciding whether someone is to get a work permit. There should be evidence, it says, that the person's employment will benefit Britain, either economically or technically, or appreciably enhance Britain's international standing in a particular field of study. The onus is on universities to convince officials that those criteria are met and that no suitable British or EEC national is available for the job.

The vice-chancellors' committee believes that the Home Office letter will help universities by providing guidelines that can be presented before immigration officials.

One further problem for universities, however, could be the Home Office's stipulation that a post must be "justifiable in its own right and not simply in terms of the candidate's abilities". In their current financial straits, not many universities are likely to be creating posts for outstanding individuals. But any that have sufficient cash should be able to hook good overseas academics simply by careful argument with the Home Office, according to David Anderson-Evans, senior administration officer at the vice-chancellors' committee.

Judy Redfearn

CORRESPONDENCE

IQ jump or trend?

SIR — Lynn¹ reported evidence of significantly higher IQ in Japan than in the United States. However, his assertion that Japanese IQ shows a "rising trend" which has resulted in a "growing disparity" is not entirely supported by his data. Statistically, a better fit is obtained by assuming that a discrete jump in IQ has occurred among Japanese born after 1945.

In his analysis, Lynn used the product moment correlation coefficient to test for trend. This implicitly supposes a linear trend. The least-squares estimate of the trend line is shown in Fig. 1 (dashed line). In fact, Lynn was prepared to discount the 1910 data point as showing the greatest discrepancy and being possibly subject to error. The trend line without the 1910 result is shown as a dotted line. Neither of these lines seems very satisfactory. In the first place, the fitted IQ for 1910 is unreasonably low, being either 98.6 or 94.7 depending upon which line is used. Second, the deviations of the data points around the lines do not show the random pattern which a genuine trend should imply. In

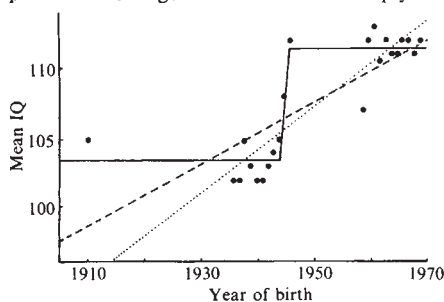


Fig. 1 Mean IQ and year of birth for 23 Japanese cohorts (data from ref. 1). The dashed line is the least-squares regression line fitted to all data points; the dotted line is the least-squares regression line fitted excluding the point for 1910. The unbroken line represents a "jump" model in which IQ is constant prior to and following 1945, but increases by a discrete step during 1945.

particular, for the 1959–69 data (mainly from a single study; see ref. 1), even though the 1959 point is low, there is little sign of a trend. If the trend was as steep as the lines suggest, it should be apparent even over a ten-year time span.

One alternative is that, instead of rising steadily, IQ has risen in a discrete jump around 1945, a time of considerable change in Japan. When the data from all cohorts are considered, the jump model (see Fig. 1) explains 89 per cent of the total variance in IQ while the linear trend accounts only for 61 per cent. If the exclusion of one outlier is permitted (jump model: 1959; trend model: 1910) these figures rise to 95 and 78 per cent, respectively. Thus, the data are much better described by a jump than by a linear trend.

Factors capable of producing a jump in the IQ results may be quite different from those which might cause a trend. In seeking reasons for the apparent rise in Japanese IQ, it may therefore be a mistake to direct too much attention to the latter.

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... as I was saying

SIR — On 6 July 1882, I sent you a letter about "The solar commercial cycle" and I promised further elucidation about the "difficulties and objections" to my theory¹. My hope was to answer my detractors who made my theory "the subject of inconsiderate ridicule"². I was prevented from doing so by the events of 13 August 1882. On that day, intending to join my family bathing at Galley Hill beach in Sussex, I chose to descend the right side of the cliff instead of the customary left side. In the cold water I suffered a stroke and my family being on the other side of the cliff were unable to come to my aid; thus I drowned.

One hundred years have passed and all attempts to prove my correlations wrong have failed. The last three to attempt it were obliged to acknowledge that my theory should be considered as a serious hypothesis^{3–5}. After more decades of silence, a few months ago two econometricians tried their new tools and also failed. They reported whimsically: "We conclude that economic activity has an important influence on the Sun. Thus Jevons ... was right but for the wrong reason"⁶.

As the acknowledged father of econometrics⁷, let me clarify the result of their test. The seemingly absurd conclusion is due to the autoregressive mechanism of the econometric model. The sunspot influence enters through the disturbance function. The model acts as a spectral filter on the now strong frequency existing in the disturbance (the sunspot cycle). But, as it is an asymmetric filter, it produces a shift in the phase of the predominant frequency, so that the output phase reading shows up *ahead* of the input reading.

I also heard lately that the American SMM (Solar Maximum Mission) satellite, kept aloft for the whole year 1980, has proved through accurate instruments that the solar "constant" varies⁸. This is very exciting to me since it confirms the hunch I had in 1878 when I suggested, in your journal, that "solar observatories should be established" high in the mountains to ask the Sun himself "whether he varies or not"⁹.

For one hundred years orthodox science has been insisting that the radiation of the Sun was a "constant". Now all must agree with my 1878 hunch: it is a variable and there are significant changes from day to day, week to week, month to month ... A spectral analysis made of the SMM satellite data suggests periodicities¹⁰, and after a few more years of data, it should be possible to unravel them for forecasting purposes.

The opposition to the factual basis of my theory will crumble some day. Stubborn anomalies cannot be kept in the scientific closet forever, for sooner or later they will out.

Concerning the linking mechanism of my theory, I have very little to add. Linking solar radiation to crop yields was the obvious first choice, but I was never satisfied with it because it did not fit the data well. I have explained how, out of sheer desperation, I thought of the psychological link: "I went so far as to form the rather fanciful hypothesis that the commercial world might be a body so mentally constituted, as Mr John Mills must hold, as to be capable of vibrating in a period

of ten years. ..."¹¹.

I mentioned this idea in all my articles in the form of an inverted rhetorical question. After so much ridicule I had to be careful. My fellow scientists had barely accepted the Darwinian theory that they had vestigial parts of lower animals in their anatomy, but my fellow economists would never accept the idea that "*Homo oeconomicus*" might not have a perfectly rational psychology and is therefore somehow at the mercy of cosmic waves.

At the end of one of my articles in your esteemed journal, I forecast that the solar constant measurement was so crucial to business that, in the future, it would be "the most important news" in the daily paper¹². Living in the Victorian era, I could only insinuate my real thoughts. It is obvious that I was not referring to the effect of these daily fluctuations on crop yields but in a direct action on the solar waves on the psychological "constant" of the population. But now, after the exciting discoveries of the SMM satellite, anybody, by reading my work carefully, can get the full extent of my intuition.

A time will come when the economists will achieve the forecasting ability of the meteorologists and the vulgar will refer to us respectfully as the practitioners of the "dismal" science, the science of calculating "*dies mali*" to advise the politician and businessman properly, not so much what to do but *when* to do it.

WILLIAM STANLEY JEVONS
(as communicated to CARLOS GARCIA-MATA)
New Canaan, Connecticut, USA

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Weapons treaties

SIR — Alastair Hay (*Nature* 8 July, p.205) confuses the international treaties on chemical and biological weapons.

The 1925 Geneva Protocol outlaws the use of both chemical and biological weapons (in practice, first use, because the major parties reserved the right to use them if attacked by them).

The 1972 Biological Weapons Convention — result of a British initiative taken in 1968 — bans the development, production and stockpiling of bacteriological and toxin weapons. The Committee on Disarmament is now discussing the elements of a similar convention banning chemical weapons, in which the UK delegation has made a major contribution with a draft treaty and a working paper on verification.

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NEWS AND VIEWS

The *myc* oncogene in man and birds

from Robin Weiss

THE HL-60 cell line derived from a patient with acute promyelocytic leukaemia¹ has proved of great importance for studies of cell differentiation and transformation. The cells in culture resemble promyelocytes but can be induced to differentiate into mature granulocytes² by treatment with dimethyl sulphoxide or retinoids. The phorbol ester tumour promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), on the other hand, causes the cells to differentiate into macrophages³. Given this capacity to differentiate along alternative pathways, HL-60 cells are also valuable for investigating natural factors operating in myeloid cell differentiation⁴ and for delineating cell surface phenotypes during maturation.

As a leukaemic cell line, HL-60 has been examined for the expression of genes which may contribute to its neoplastic phenotype. Weinberg's laboratory⁵ showed that HL-60 DNA sequences transfected into NIH/3T3 cells caused transformation. The transfecting gene remains to be characterized. In the meantime the organization and expression of the *c-myc* gene in HL-60 cells has become a focus of interest.

The *myc* gene was first defined as the unique transforming sequence of MC29 virus and related retroviruses which cause myeloid neoplasms and other types of tumour in chickens⁶. Like other transforming genes ('oncogenes') of retroviruses, DNA sequences homologous to *myc* are found in normal chicken DNA⁶. The viral oncogene, *v-myc*, thus represents a transduced cellular gene, *c-myc*. As *c-myc* sequences have been highly conserved in evolution⁷, a cDNA probe prepared from the avian viral oncogene can be used to detect and clone the homologous gene in human DNA and to study its expression. The *c-myc* gene is expressed in a variety of neoplastic and proliferating normal human cells⁸, but mRNA levels appear to be unusually high in HL-60 cells⁹.

Two laboratories have now shown that *c-myc* DNA sequences are amplified in HL-60 cells. Collins and Groudine¹⁰, using an avian *c-myc* probe, reported in *Nature* a few weeks ago that HL-60 cells have about

16 *c-myc* copies. In this issue of *Nature* (p.61) Dalla Favera *et al.*¹¹, using cloned human *c-myc* probes, confirm this finding and show, moreover, that the leukaemic cells of the patient from whom HL-60 cells are derived were similarly amplified for *c-myc* before the cell line was established in culture. Unfortunately, normal tissue from the patient (long since dead) is no longer available so that it cannot be established whether the *c-myc* gene amplification was constitutional or was the result of somatic mutation in the leukaemia cell clone. Dalla Favera *et al.*¹¹ report that closely related pseudogenes are not amplified, suggesting that amplification might result from selection of *c-myc* function in the leukaemia cells. However, the few other patients with acute myeloid leukaemia that have been studied do not have amplified *c-myc* sequences.

Amplification of other oncogenes has been observed, but in species rather than tumours. Two related cellular genes homologous to sarcoma virus oncogenes, *Ha-ras* and *Ki-ras*, are found in markedly different copy numbers in closely related species¹². For instance, *Mus pahari* contains at least 10 copies of *c-Ha-ras*, whereas other species of mice carry only 1 or 2 copies. The transfecting gene of the human bladder carcinoma line, EJ, is now known to be the *c-Ha-ras* gene¹³, but it is not amplified in the tumour cells. It has not yet been shown whether the transfectable gene in HL-60 cells⁵ is *c-myc*; it may well be a different gene. In chicken bursal lymphomas, although *c-myc* expression is specifically elevated by adjacent provirus integration¹⁴⁻¹⁶, the gene identified by DNA transfection is quite distinct¹⁵. Similarly, the SMS-SB human pre-B leukaemia line expresses high levels of *c-abl*¹⁷, but a different transforming gene has been detected by transfection¹⁸.

The *myc* gene of MC29 is expressed in infected cells as a *gag-myc* 'fusion' protein, p110, translated from a transcript of part of the viral *gag* gene running into

myc. This was first recognized when *gag* antisera precipitated p110 from extracts of transformed cells¹⁹. Following this discovery, several other *v-onc* genes of replication-defective, transforming viruses were also found to be expressed as *gag-onc* fusion proteins. Avian retroviruses of the MC29 group, which carry *v-myc* genes, cause a bewildering variety of neoplasms — myelocytomas, endotheliomas, mesotheliomas, renal and hepatic carcinomas and sarcomas. In some elegant experiments, Graf *et al.*²⁰ showed that MC29 virus transforms both fibroblasts and macrophages in culture, but not erythroblasts, even though p110 is synthesized in infected erythroblasts. Thus, despite the variety of tumours associated with MC29 virus, there is clearly some specificity of target cells for neoplasia following infection. Furthermore, the disease spectrum can be restricted by selective passage *in vivo* of virus extracted from one type of tumour, for example, bone marrow or liver²¹, or by the isolation of *v-myc* deletion mutants *in vitro* that maintain their capacity to transform fibroblasts but are unable to transform macrophages²².

Lymphoid neoplasms are not usually caused by MC29 virus, yet the activation of *c-myc* by provirus integration in the vicinity of the cellular gene is evident in the majority of bursal lymphomas induced by avian leukosis viruses¹⁴⁻¹⁶ and also by the unrelated helper virus of reticuloendotheliosis virus²³. It is puzzling that the elevation of *c-myc* expression by the insertion of viral regulatory sequences appears to be specific to B-cell neoplasms, whereas *v-myc* inserted as part and parcel of the viral genome primarily induces other types of cancer. Hayman *et al.*²⁴, however, have now found that a transforming revertant of one of their *v-myc* deletion mutants causes a high incidence of lymphoid neoplasms with short latency. Tryptic peptide analysis of the *gag-myc* protein of this revertant indicates that the original deletion mutant may have recombined with *c-myc*. The data on deletion mutants and revertants^{22,24}, together with the evidence of selection of disease-specific strains of MC29²¹, suggest that mutations

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in the *myc* gene affect its target cell specificity for neoplastic transformation.

What, then, is the function of the p110 protein encoded by the *gag-myc* gene? Unlike several *v-onc* products, p110 has not been shown to have tyrosine phosphokinase activity. Recently, Moelling's²⁵ and Eisenman's²⁶ laboratories have shown both by subcellular fractionation and by immunofluorescence with *gag* antisera that the *gag-myc* protein p110 is located in the nucleus of transformed, non-virus-producing quail cells. Furthermore, purified p110 behaves as a DNA-binding protein²⁵. Since other *gag*-related polypeptides do not migrate to the nucleus or bind to DNA, it is thought that these properties relate to the transforming, *myc*-specific domain of the protein. It will be interesting, therefore, to ascertain whether the *gag-myc* proteins of the MC29 mutants²² bind to chromatin in both fibroblasts (which they transform) and macrophages (which they cannot transform), and the location and function of *c-myc* products, especially of the amplified genes in the HL-60 human leukaemia cells, also merits investigation.

The MC29 *gag-myc* protein is not the only transforming protein to have a nuclear location and DNA-binding properties. Cells transformed by Epstein-Barr virus, adenovirus and papovaviruses all express virus-coded nuclear antigens. As reviewed recently in these columns²⁷, two different viral proteins, SV40 T-antigen and a 58,000 molecular weight protein of adenovirus, both have a high affinity for p53, a host nuclear protein which may play a role in cell proliferation. Could the *myc* product be a transforming protein analogous to T-antigen or p53? □

Streptomyces in the ascendant

from K.F. Chater

DESPITE their manifest importance in antibiotic production, differentiation and soil ecology, mycelial Gram-positive bacteria of the genus *Streptomyces* have been little studied by geneticists and molecular biologists. That this situation is about to change was perhaps the major message conveyed in a recent symposium* in Kyoto on the genetics of these and many other industrial microorganisms. Various novel genetic phenomena in *Streptomyces* were documented, and the availability and use of sophisticated *in vivo* and *in vitro* *Streptomyces* recombination systems, and first steps in understanding both homologous and heterologous expression of *Streptomyces* genes were described.

A + T-rich repeated sequences (up to 12 per cent of the total DNA) are widespread in streptomycetes (R. Kirby *et al.*, University of Capetown). These sequences may have roles both in certain cases of chromosomal and plasmid structural instability, which sometimes involve the complete loss of particular DNA segments such as the tyrosinase gene of *S. reticuli* (H. Schrempf, University of Wurzburg), and in the sporadic extreme tandem amplification of functionally uncharacterized DNA sequences (H. Ono *et al.*, Institute of Microbiology, Zürich; H. Schrempf and see ref. 1). Equally unusual is the discovery of linear *Streptomyces* plasmids which have identical 611 base pair sequences at both ends of the 17 kilobase kb DNA molecules and proteins covalently bound to the 5' termini (H. Hirochika *et al.*, Mitsubishi-Kasei Institute of Life Sciences, Tokyo).

Whether it will be possible to exploit these phenomena in the laboratory remains to be seen. Even without them, the *Streptomyces* geneticist has a formidable range of tools, now that endogenous *Streptomyces* DNA cloning is becoming routine. One of the most sophisticated vectors, pIJ61, which is based on the low-copy number plasmid SLP1.2, contains a selectable thiostrepton-resistance marker and a very efficiently expressed neomycin phosphotransferase gene with sites for insertional inactivation (C. Thompson *et al.*, John Innes Institute). pIJ61 was used in what is probably the first cloning of a gene involved in antibiotic production: a *para*-aminobenzoic acid synthetase gene (*pab*) involved in candidin biosynthesis in *S. griseus* (J. Gil and D. Hopwood, John Innes Institute). Although expressed in several streptomycetes, the cloned *pab* gene was not expressed in *E. coli* unless placed under the control of an *E. coli* promoter by a deletion (it could then 'complement' mutations in both of the

unlinked *pabA* and *pabB* genes of *E. coli*). Several other *Streptomyces* genes failed to be expressed from their own promoters in *E. coli*, including genes for neomycin and viomycin phosphotransferases (D. Hopwood).

Homologous and heterologous gene expression involving *Streptomyces* DNA has been more specifically analysed with a promoter-probe vector also based on SLP1.2 (M. Bibb and S. Cohen, Stanford University). Promoters from *E. coli* (*lacUV5* and *recA*), *Serratia marcescens* (*trp*) and *Bacillus* (*penP*) all allowed the expression in *Streptomyces* of a promoterless chloramphenicol acetyltransferase gene from *E. coli*, as did a number of *Streptomyces* small DNA fragments. On the other hand, the latter fragments did not promote transcription in *E. coli*, their DNA sequences being quite unlike consensus *E. coli* promoters: their only consistent feature was a relative A + T richness (about 60 per cent G + C) in the sixty or so bases upstream of the translated regions, compared with the average G + C content of 70–73 per cent for *Streptomyces* DNA. From these results and other descriptions of the expression of R-primes among various Gram-negative bacteria (B. Holloway, Monash University, Australia) one gains the impression that promoters from low G + C DNA express in organisms with higher G + C DNA more effectively than the reverse. Together with other evidence from Bibb and Cohen that there may be less specificity for ribosomal binding to mRNA in *Streptomyces* than in *Bacillus*, streptomycetes may be uniquely suitable for studies involving heterologous prokaryotic gene expression. No normal vegetative *Streptomyces* promoters have yet been analysed, though examples of these should soon be available for study, since highly regulated *Streptomyces* β -galactosidase and glycerol degradation genes have now been cloned (T. Eckhardt and L. Fare, Smith, Kline & French Laboratories, Philadelphia; E. Seno *et al.*, John Innes Institute). As a result, antibiotic (methylenomycin A) production genes have been cloned in an attachment site deleted ϕ C31 vector carrying a viomycin resistance gene. The cloned genes were recognised because they allowed the vector to integrate into, and disrupt, the homologous genes present in a recipient, giving viomycin resistant, methylenomycin non-producing colonies (K. Chater *et al.*, John Innes Institute).

Among several λ -like *Streptomyces* phage vector systems, the most advanced is based on phage ϕ C31. An early difficulty of rather low transfection frequencies has

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*The Fourth International Symposium on Genetics of Industrial Microorganisms, Kyoto, June 6–11 1982, was the sequel to earlier meetings in Prague (1970), Sheffield (1974) and Madison (1980).

Stamp watching and bird collecting

from Robert M. May and Judith May

AESTHETICALLY and philatelically the recent issue of US bird-and-flower stamps is remarkable; each sheet has 50 different 20 cent stamps, depicting the 'state bird' and 'state flower' of each of the 50 states. Scientists may find them additionally interesting for the way they exemplify the peculiarity of our engagement with the order Aves.

Knowing nothing about how the state birds were originally chosen, and given that there are 700 or so species to choose from, one can generate all manner of hypotheses about the diversity of bird species to be found on this set of stamps. The 'null hypothesis' would be that the states chose their birds independently and randomly, in which case we would expect only one or two pairs of states to have chosen the same bird species, and the remaining 48 or 46 states each to have made a unique choice. Of course, this null hypothesis is obviously too simple, as it overlooks biological complications in the relative abundance and geographical distribution of birds, historical and cultural factors in the founding of states, and the particular aesthetic attractions of the favoured species. The stamps in fact show the state birds to be a much less diverse assembly than would be expected on any biological grounds: 7 cardinals (for states from the east coast to Kentucky and Indiana); 6 western meadowlarks (mainly in the north-west); 6 mockingbirds (from southern states); 3 robins; 2 mountain bluebirds; 2 eastern bluebirds; 2 American goldfinches; 2 chickadees; and only 21 singletons.

Over the years, scientists too have shown that they are not immune to avian charms (though the sheer conspicuousness of birds, in comparison with most other vertebrates, undoubtedly accounts for some of this popularity). From Gilbert White's time onwards, a grossly disproportionate number of evolutionary and

ecological studies focus on birds. Many, if not most, of the subject's leading theoreticians in this century — Mayr, Lack, MacArthur and others — have been 'bird people'.

A more general reflection of this fascination is to be found in the hobby/sport/obsession of bird watching, and especially in its strange outgrowth of bird listing. We suppose some people keep life lists of the species of mammals or flowers, though surely not of insects, they have seen (after all, there do exist train listers and plane listers), but we have not met any. Bird listers, however, abound.

From their point of view, North America is a richer place than Britain; around 660 species of birds breed in North America (excluding Greenland, Bermuda and Baja California) and around 710 species are regularly seen, whereas only around 190 species of birds breed in Britain and only around 240 species are regularly seen. This difference in the number of breeding bird species is almost exactly what would be expected from the MacArthur–Wilson species–area relation, $S \sim A^z$, with $z \sim 1/4$; the ratio of the area of North America to that of Britain is roughly 80, whence we would expect roughly a ratio of 3 in the number of species. The agreement is surprising, and must surely be largely coincidental, because the underlying premises from which the species area relation is deduced pertain more to archipelagoes of islands than to large continental masses.

The list of species ever seen in North America totals around 820. In Britain the list numbers around 470, many of which are rare sightings of birds that are lost and do not want to be there. From these numbers it can incidentally be seen that membership in the '600 club' (a life list of 600 North American birds) is a substantial but not unreasonable goal. More arcane are competitions centering on the greatest number of bird species sighted in North America in one year, or simply the greatest number of species seen in any one day, or even by turning through 360° on one spot. The record for North American bird species seen in one calendar year was until recently held by Scott Robinson, now a Princeton University graduate student, at 657. In 1979, a businessman named Vardaman spent a small fortune in pursuit of the ambition of sighting 700 species in one year. Travelling extensively and advertising widely for people to lead him to rare birds, Vardaman pushed the total number of species he had seen above 690 in the last weeks of December. The magic 700 was to elude him, however. At the year's end Vardaman's record stood at 699. God too is clearly an amateur bird lister.

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been overcome by the surprising (and perhaps more widely applicable) finding that small positively charged, DNA-free liposomes greatly stimulate polyethylene glycol-assisted transfection of protoplasts (J. Makins, *et al.*, John Innes Institute).

Cloning systems are especially useful when allied with *in vivo* genetics. This has been made easier in *Streptomyces* by several recent developments. For example, the multicopy 8.9 kb plasmid pIJ101 is a very efficient sex factor which can be transferred into nearly all streptomycetes (D. Hopwood). Other generalized tech-

niques for achieving efficient chromosomal recombination in *Streptomyces* include protoplast fusion² and liposome-mediated transformation³. Perhaps less widely applicable is a system for Ca^{2+} -promoted transformation of competent *S. griseus* cultures by plasmid DNA (Z. Zhuang *et al.*, Institute of Microbiology, Beijing) and the discovery of a (probably narrow host range) temperate phage of *S. fradiae* which exists as a plasmid prophage and is capable of generalized transduction (S. T. Chung, The Upjohn Company, Kalamazoo).

Evidently the immediate future will see the detailed analysis of primary and secondary metabolism and differentiation in *Streptomyces*, together with a widening of our vision of prokaryotic genome structure. Some of the new techniques are sure to influence industrial programmes and may lead to the use of streptomycetes as hosts for foreign DNA expression. □

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Seismic triggering and earthquake prediction

from W. Thatcher

WHILE identification of seismic gaps and the determination of earthquake recurrence intervals each provide viable means of assessing long-term earthquake potential¹, at present no comparably reliable methods exist to evaluate intermediate or short-term earthquake risk. Recent work, however, suggests that estimation of short-term risk might be improved by taking into account the potential of aseismic deformation episodes for triggering large earthquakes and incorporating this into a probabilistic assessment of earthquake risk.

The notion that aseismic movements could trigger damaging earthquakes is by no means new. Ever since Richter² pointed out the rather regular east-to-west migration of large shocks on the North Anatolian fault in Turkey, seismologists have been proposing a variety of aseismic processes that might explain this pattern and features similar to it³. What is new, however, is the accumulating evidence that transient and episodic aseismic movements are relatively common rather than extremely rare, and in several well-documented instances it appears that such movements have indeed triggered large earthquakes. The purpose of this article then is two-fold: first, to briefly review the new evidence, and second, to point out how it suggests a more integrated approach to earthquake prediction, one that draws on the evidence of studies of artificially-induced seismicity and that incorporates recently-proposed methods for the probabilistic assessment of earthquake risk⁴.

Although it has been commonly assumed that stress in seismically active regions builds up at uniform rates, geodetic survey measurements provide the evidence that this is not the case. Transient post-seismic movements of considerable size may occur over large areas for several decades after great plate boundary shocks^{5,6}, and episodic strain or elevation changes have been detected in closely monitored seismic gaps in southern California and Japan⁷⁻⁹. Uniform and non-uniform models of stress buildup are contrasted in Figure 1 and it can be seen that episodic movements could trigger a large earthquake to occur sooner than it otherwise might. Figure 2 suggests how episodic deformation could lead to the occurrence of a discernible increase in pre-earthquake minor seismicity.

Both observations and simple triggering models agree that large earthquakes can actually occur as a result of the episodic aseismic stress changes seen in geodetic

survey observations. The best evidence comes from regions where volcanic activity occurs in association with large tectonic earthquakes and triggering is the result of stress changes due to subsurface movements of liquid rock (magma) and associated gases^{10,11}. The relation between volcanism and crustal deformation on the one hand and seismicity and large earthquakes on the other is particularly well seen in Japan's Izu Peninsula¹¹.

There, two episodes of domal uplift have taken place this century (1930-33 and 1975-82), each was centred in a region of

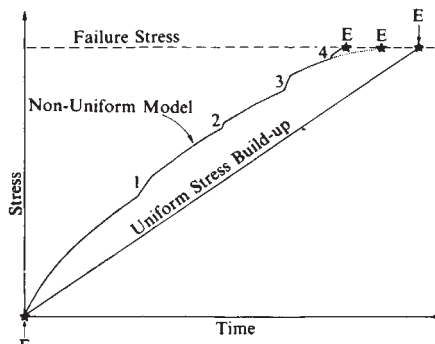


Fig. 1 Idealized modes of stress (or strain) accumulation between consecutive large earthquakes (E). Nonuniform model includes effects of both a steady postseismic decline in rate and the random occurrence of short periods of greater-than-average rates of stress increase (1-4), the last of which triggers seismic failure sooner than would otherwise have been the case (dotted curve). For simplicity the figure assumes that stress levels at any instant are everywhere the same on the eventual rupture surface. Alternatively, it can be considered that the figure shows only the stresses at the eventual earthquake hypocentre, the initiation point of failure.

Quaternary volcanism, involved uplifts of 0.2 m or more, and covered an area about 30 km in diameter. A simple magma-chamber-inflation model explains the uplift quite well¹². Near the periphery of the uplifted regions, three strike-slip earthquakes of about magnitude 7 have occurred (1930, 1978, 1980) and been preceded by one to three years of domal uplift in an adjacent region. In addition, earthquake-swarm activity was particularly marked during 1930-34 and 1975-81 and conspicuously absent during the intervening 40 years.

An inflation model shows¹¹ that shear stress increments of about a bar (0.1 MPa) were generated across the fault planes of each of these three earthquakes, in all cases acting in the correct sense to trigger seismic slippage. Of course, this increase is no more than a few per cent of the stress increment required to cause failure, the majority being supplied by regional tectonic stresses resulting from the collision of the Philippine Sea and Eurasian plates¹³.

However, because the recurrence interval for earthquakes of magnitude 7 on each of these faults is approximately 1,000 years or more¹⁴, these inflation-induced stress changes represent the equivalent of several decades of secular average stress buildup.

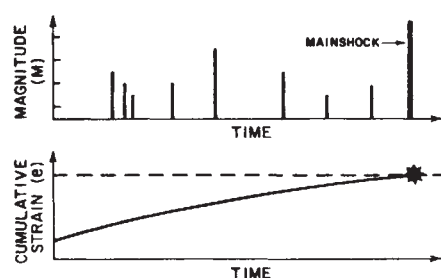
Several studies suggest that postseismic transient stress changes trigger notable earthquake activity. Utsu and Shimazaki¹⁵ have shown that in Japan, large intraplate earthquakes occur more frequently during the 10 years after a great underthrust event at the adjacent plate boundary than at other times. Studies of postseismic level changes that followed the great 1946 Nankaido earthquake demonstrate that, in contrast to interseismic secular movements, transient deformation in the several decades since 1946 extended more than 350 km inland from the plate boundary⁶. In addition, Lehner *et al.*¹⁶, following a suggestion by Anderson¹⁷, have shown that observed migrations of great earthquakes along major plate boundaries can reasonably be attributed to gradual lateral diffusion of postseismic stress away from the rupture zones of individual shocks.

Earthquakes induced by reservoir loading provide a further example of seismic triggering consistent with the mechanism sketched in Figure 1. As the graph suggests, episodic stress changes, of which the filling of a reservoir is one example, need not trigger damaging earthquakes, or indeed any earthquakes at all. Should they do so, the triggering stresses need only be a small fraction of the ambient tectonic level. Simpson¹⁸ has shown that discernible increases in post-impoundment seismicity have occurred in only a small proportion of the world's large reservoirs but further suggests that in many instances only very small stress or pore pressure changes were required to induce tectonic earthquakes of magnitude 5 or greater. Earthquakes associated with fluid injection were probably triggered by considerably larger pore pressure changes¹⁹. Swarm activity appears to precede all the well-documented large artificially-induced shocks¹⁸, and the mechanism sketched in Figure 2 may be an appropriate explanation of this activity.

While not being definite indicators of impending earthquakes, the occurrence of episodic deformation can nonetheless be taken as one indication of increased seismic risk, and several studies have suggested how they might be used to assess earthquake hazard. With respect to the strain changes observed in southern California during 1979, Raleigh *et al.*²⁰ draw attention to the non-linear character of the buildup occurring there and suggest that risk be assessed by determining whether the strain increments bring the faults of the San Andreas system closer to failure or tend to

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① UNIFORM STRAIN BUILDUP



② EPISODIC DEFORMATION

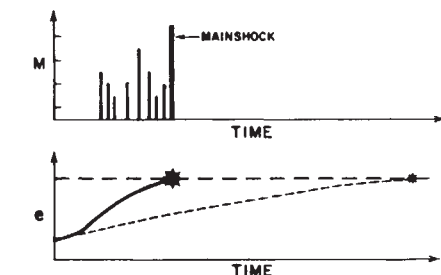


Fig. 2 Possible forms of pre-earthquake seismicity.

lock them, impeding slippage. They further emphasize the importance of concurrent monitoring of seismicity levels on these faults. In evaluating an earlier episode of straining that may have been related to the southern California uplift⁹, I suggested²¹ that the pattern of movements indicated relative locking of the San Andreas fault but increasing shear stresses on the bounding thrust faults of the Transverse Ranges.

Evaluation of these and other factors, as well as more concentrated monitoring, should provide rational criteria for deciding whether the probability of an earthquake has increased or decreased. Once a deformation episode has been identified, a discernible increase in micro-seismicity would provide one indicator of increased risk. The magnitude and sense of stress or strain change across candidate faults would provide another basis for deciding whether risk level should be raised or lowered. Provided areal and temporal coverage were adequate, such changes could be determined directly from geodetic data; alternatively, as was the case for the Izu Peninsula earthquakes¹¹, model calculations or indirect inferences might suffice. Earthquake focal mechanisms provide a third evaluation criterion: if micro-fault movements are in the sense expected from observed or inferred stress increments, then the probability of a damaging shock should be correspondingly increased. Even at this point, however, a large earthquake remains by no means inevitable and, in this view, no amount of positive evidence will suffice to make it so.

Nonetheless, this monitoring strategy has several advantages. It is straightforward, relies on simple physical concepts, and emphasizes well-proven methods of geodetic surveying and seismic

network operation. An emphasis on identifying episodic crustal movements and their consequences provides a rationale and focus that may be absent in a strictly empirical search for earthquake precursors. Once anomalous movements are detected, follow up studies can begin immediately and provide risk assessments that can be continuously updated.

The ultimate importance of triggering mechanisms for earthquake prediction depends, of course, on how commonly large earthquakes are caused by detectable episodic or transient stress increases. Although this is not known, in general the probability of triggering would be expected to increase as the frequency and amplitude of episodic strain changes increase. Clearly, as Figure 1 indicates, the fault zone must already be rather close to failure.

Obvious candidates for close attention are identified seismic gaps and faults for which the time since the last large earthquake is comparable to the recurrence interval for such events; Matsuda¹⁴ has identified four such intraplate faults in Japan and refers to them as 'precaution faults'. McCann *et al.*¹ defined category 1 seismic gaps as those likely to rupture in a great earthquake 'within the next decade or few decades', and showed that seven gaps identified by this criterion since the late 1960's have subsequently been filled. From this perspective, the potential importance of triggering can perhaps best be appreciated by noting that, for many such seismic gaps, already documented episodic movements would be the equivalent of a decade or more of secular strain accumulation. □

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Synthetase U-turns

from Graham Cowling

Now that the problems of gene sequence and structure are essentially solved, molecular biologists are beginning to tackle the next obstacle to a complete understanding of the translation, transcription and replication processes — the interactions between proteins and nucleic acids. The task is clearly a Herculean one and it is thus encouraging to find that advances have already been made in one area, the mechanism of aminoacyl-tRNA synthetase action.

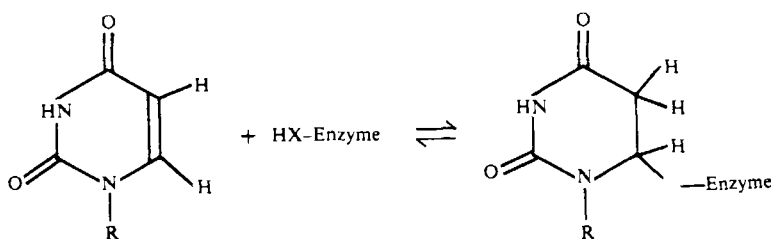
The aminoacyl-tRNA synthetases are an abundant class of cellular enzymes that assign amino acids to trinucleotide sequences (anticodons) of tRNA. They aminoacylate tRNA in a two-step reaction; first an amino acid is condensed with ATP to form an enzyme-bound aminoacyl adenylate complex and then the complex reacts with tRNA to give aminoacyl-tRNA. It is thought that there

are at least as many individual synthetases as types of amino acid and each enzyme recognizes several isoaccepting tRNAs. While each synthetase catalyses the same general type of reaction, they vary in size and quaternary structure.

The catalytic mechanism of aminoacyl-tRNA synthetase requires at least four steps; aminoacyl adenylate formation, recognition of cognate forms of tRNA, binding of tRNA to the enzyme and transfer of amino acid to tRNA. Recent work from Schimmel's group¹ has shown that at least two of these steps are related by demonstrating that a covalent bond between uridine 8 of *Escherichia coli* tRNA^{Ala} and its respective synthetase appears to be linked with enzyme activity. They further suggest that this covalent bond may be essential for active enzyme conformation.

While tRNAs vary considerably in length and nucleotide sequence, all cytoplasmic tRNAs have uridine (or 4-thiouridine) at position 8 of the nucleotide chain. This residue lies on the

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Michael addition between a synthetase nucleophile and the uracil ring

inside of the vertex made by the tertiary L-shaped structure of tRNA and is well positioned to interact with synthetase. Previous observations suggest that synthetase binds tRNA by a transient, covalent bond between a uridine residue and a nucleophilic site on the protein. Synthetases seem able to catalyse the exchange of hydrogen atoms at C-5 of uridine 8 in cognate tRNAs; a reaction which involves nucleophilic attack by the enzyme on C-6 of the uracil ring to form a Michael addition product. Reversal of the reaction results in the observed H-5 exchange (see the figure). Similar mechanisms involving the formation of 5, 6-dihydropyrimidine intermediates are common with enzymes that modify pyrimidine nucleotides. Examples include thymidylate and pseudouridylate synthetases, dUMP and dCMP hydroxymethylases and DNA methylases.

Understanding of the action of thymidylate synthetase has been much helped by the use of uridine substrates with electron withdrawing groups at C-5. The substitution increases the susceptibility of C-6 to nucleophilic attack and thus allows the isolation of stable nucleoside-enzyme intermediates. This strategy has, however, failed in the case of aminoacyl-tRNA synthetase because the enzyme catalyses the breakdown of 5-bromouridine to give pyrimidine base; ribose and free enzyme. Schimmel *et al.* solved this problem by finding conditions that increase both the formation and stability of 5-bromouridine-synthetase complexes. Experimentally, this involved using a greater excess of uridine derivative and omitting sulphhydryl reducing agents in the reaction. As a result a stable 'dead-end' complex between 5-bromouridine and synthetase accumulated. Each subunit of the tetrameric enzyme was found to bind one molecule of 5-bromouridine. If the site on synthetase which interacts with either 5-bromouridine or uridine 8 in tRNA is required for activity, binding the uridine derivative should block the binding of tRNA and destroy the aminoacylation activity. This seems to be the case and suggests a single uridine binding site on the enzyme.

E. coli Ala-tRNA synthetase is composed of four identical subunits each of 875 amino acids. It had already been shown that the N-terminal half of each chain (440 amino acids) is responsible for aminoacyl adenylate synthesis but cannot catalyse the

aminoacylation of tRNA². Schimmel and co-workers further demonstrate that the uridine binding site is at the C-terminal end of synthetase and 5-bromouridine does not interfere with aminoacyl adenylate formation. Data from segment directed mutagenesis experiments (unpublished) indicate that only a relatively short stretch of 30–60 residues in the C-terminal end of synthetase is required for effective tRNA binding.

Other recent studies point to a conformational change being part of the synthetase mechanism. Ebel and co-workers³ found that yeast Phe-tRNA synthetase was able to aminoacylate adenosine mononucleotide if the enzyme was conformationally triggered by binding defective yeast tRNA^{Phe} which has been stripped of its 3'-terminal adenosine (the normal site of aminoacylation). Baltzinger and Holler⁴ also concluded after kinetic studies of the catalytic mechanism of *E. coli* Phe-tRNA synthetase that a conformational change of

the nascent enzyme-tRNA complex is the rate limiting step. Whether the covalent bond between the synthetase and uridine 8 of tRNA plays a role in catalysis is less clear.

Schimmel *et al.*⁵ speculate that the formation of such a bond may allow the domain responsible for adenylate formation to interact with the bound 3'-end of the tRNA. If this mechanism is correct, it will be interesting to discover how synthetases recognize the differences between cognate and non-cognate tRNAs. Although non-cognate tRNAs have been shown to bind to synthetase, this does not result in aminoacylation. Non-cognate tRNA mixtures also fail to undergo synthetase-mediated H-5 exchange. Assuming uridine binding does not occur with non-cognate tRNA, the synthetase must use other sequence or spatial criteria as part of its tRNA recognition step. These questions, as yet, remain unanswered. The abundance and heterogeneity in size and structure of synthetases suggest that the discovery of further diverse cellular functions will ensue. Already *E. coli* Ala-tRNA synthetase has been shown to bind to *alaS* promoter sequences, acting as a gene repressor of alanine biosynthesis⁶. □

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The first meteorite stream

from David W. Hughes

METEOROID streams are common enough. Comets decay and their dust slowly spreads around the orbit forming a stream of particles in space that can be detected if the Earth intersects the stream. The August Perseids, December Geminids and January Quadrantids are examples of the displays of shooting stars produced by these intersections. The dust particles responsible usually have masses below a few grammes.

In contrast, meteorites, the rock and iron bodies that fall to the earth's surface, are much more massive and have been thought to reach the earth not in streams, but at random. Now scientists are having doubts. Seismometers left on the lunar surface by the Apollo 12, 14, 15 and 16 missions can detect meteorite impacts and as many as 150 have been recorded in a single year. Far from being random it seems that the impact rate varies throughout the year and increases between April and July.

Charles A. Wood of the Johnson Space Centre, Houston, is convinced that meteorite streams can be detected on Earth

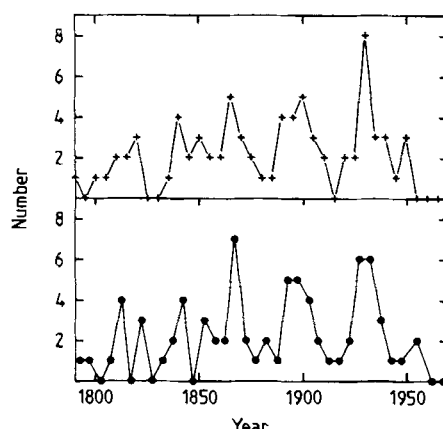
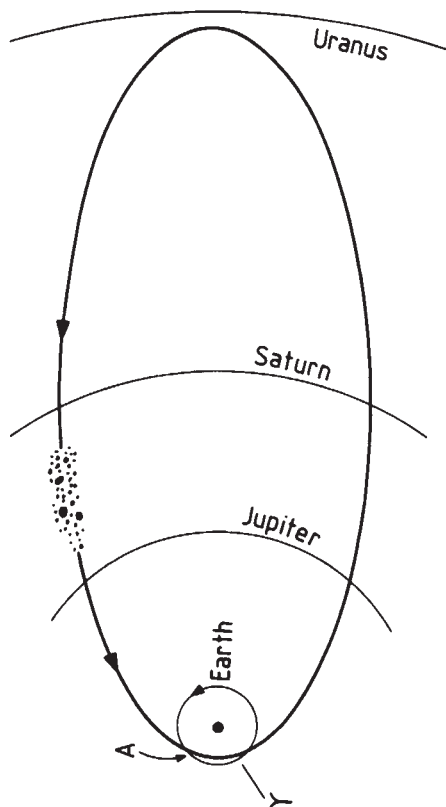


Figure 1. The number of H chondrites with cosmic ray exposure ages between 2 million and 8 million years that have been seen to fall to the Earth in a five year interval. The top data set has typical intervals 1825.5 to 1827.5, the lower data set has typical intervals 1820.0 to 1825.0. A frequency periodicity of 31.2 years can be seen with maxima in 1813, 1838, 1867, 1899 and 1929.

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and has published his findings in *The Proceedings of the XIII Lunar and Planetary Science Conference* 873; 1982. Wood has selected from a specific class of meteorites, ordinary chondrites with a high iron content (H chondrites), those with exposure ages between 2 and 8 million years. Cosmic rays bombarding the meteorites in space leave tracks of radiation damaged material analogous to the tracks produced by fission. Analysis of these tracks leads to an exposure age that for H chondrites peak around 4.45 ± 0.86 million years. It is generally assumed that H chondrites were part of a larger body which fragmented at the time. The falls of H chondrite in this group (figure 1) are clearly clumped in time. Noether's test for cyclical trends and the Kolmogorov-Smirnov test both confirm that the distribution is highly non-random. Power spectrum analysis gives a frequency period of 31.2 years. Maxima occur in 1813, 1838, 1867, 1899 and 1929.

The 31 year periodicity immediately indicates that the meteorites are clumped in space, in the early stages of forming a meteorite stream. An analogy is the Leonid meteoroid stream which has a period of 33 years, the small dust particles still being close to the parent comet. A period of 31 years corresponds (due to Kepler's Law) to a semi-major axis of 9.9 AU and, as the meteorites hit the Earth, it can be assumed that the perihelion distance is about 0.9 AU. The resulting orbit is shown in Figure 2. Meteorites on this orbit will hit the Earth at a specific time each year. The time of year of the fall of the meteorites from the three

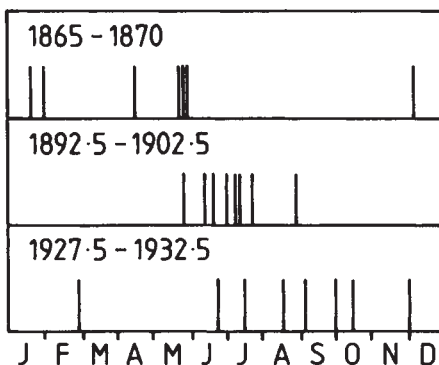


Fig. 2 The meteorite stream is shown to the left. Meteorites hit the Earth at A, during June and July at a relative velocity of about 17 km s^{-1} . Impacts occur about every 31 yrs. γ represents the first point of Aries.

In the chart above the bars represent the time of fall of the meteorites that impact at the peaks of the flux curves given in Figure 1. There is a hint that the maximum flux occurs around June and July.

rate maxima are shown in the chart in figure 2. Obviously stream and sporadic meteorites are included here but still there is a hint that the maximum flux occurs around June and July, which happily agrees with the lunar seismic data. Meteorites in the orbit shown in figure 2 hit the Earth with a geocentric velocity of about 17 km s^{-1} so a reasonable amount of the meteorite survives the retardation in the atmosphere and falls to the surface.

Two major problems still await a solution. The first is the definition of the expected 1960 peak. Only 8 out of the 26 H chondrites falling between 1955 and 1970 have had their exposure ages determined.

The second problem concerns the orbit shown in figure 2. Conventional wisdom has meteorites originating from main belt asteroids. The solar gas content of

meteorites seems to require a long residence time in the inner solar system. A parent body in the asteroid belt, where about 10 per cent of the mass can be converted into regolith in 4.5×10^9 yr, is ideal (see Anders, E., *Icarus* 24, 363; 1975). Short period comets absorb too little of the solar gases and can thus be ruled out as parent bodies, at least for all stony meteorite classes that have gas-rich members. Unfortunately, there are dynamic problems in getting meteorites from the main asteroid belt. Also, infrared observation of asteroids reveal an embarrassing lack of unambiguous parent bodies for the ordinary chondrites. Around 80 percent of terrestrial meteorites are in this class. The orbit shown in Figure 2 however, is most unasteroid like and is typical of short period comets. It also has an aphelion distance similar to Chiron, the anomalous object orbiting between Saturn and Uranus.

Charles A. Wood and W.W. Mendell (*The Proceedings of the XIII Lunar and Planetary Science Conference* 877; 1982) conclude that the young H chondrite orbit is unstable and is representative of an orbital stage in the evolution of a small body which is moving inwards in the solar system. They propose that the H chondrite parent body was formed in the inner solar system, was ejected by Jupiter during the accretion epoch and has recently been pulled out of the Oort cloud to be reperturbed into a 'comet like' orbit that also intersects the Earth's orbit. The collision suffered by the parent body 4.45 million years ago gave the fragments very low velocities with respect to the centre of mass. This has resulted in the small spread of meteorites around the orbit and the clear indication of a 31 year period in the influx rate. The degree of spreading is shown by the narrowness of the peaks in Figure 1. □

Autoimmunity and the aetiology of insulin-dependent diabetes mellitus

from C. Ronald Kahn

ALTHOUGH their cause remains unknown, evidence has accumulated over the past decade that the two common types of diabetes, insulin-dependent diabetes mellitus (IDDM; also known as type I or juvenile-onset diabetes) and non-insulin-dependent diabetes mellitus (NIDDM; also known as type II or maturity-onset diabetes) may be aetiologically distinct. The damage found in IDDM appears to result from an autoimmune process which is directed at the insulin-producing β cells of the pancreas and ultimately leads to their destruction and the development of the

syndrome. The autoimmune process is probably triggered by some environmental factors in a genetically susceptible individual — exactly what these factors are is unknown but animal studies suggest viruses or toxins are likely candidates.

The first clues that an immune mechanism was involved in diabetes came from the association between IDDM and other endocrine deficiencies that had an autoimmune basis, such as those of the adrenal or thyroid glands¹. Patients with IDDM were also seen to have a remarkable increase in the prevalence of other organ-specific autoantibodies in their sera.

Direct evidence for cell-mediated autoimmunity directed against the endocrine

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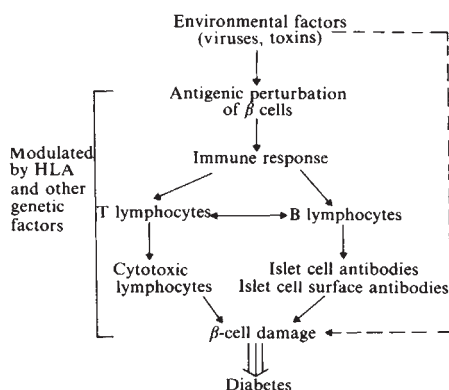
pancreas was provided in 1971 when it was found that the migration of leukocytes from patients with IDDM was inhibited by exposure to antigens prepared from endocrine pancreas². Diabetic lymphocytes also have increased adherence and cytotoxicity against cultured insulinoma cells (tumour cells of the insulin-producing β cells in the islets of Langerhans — usually benign)³. The changes in function are accompanied by alterations in the circulating lymphocyte populations in acute IDDM in both man and the BB rat, which develops an IDDM-like syndrome⁴. The passive transfer of diabetes from man to mouse and mouse to mouse using affected lymphocytes has also been reported⁵, but has been challenged as a number of groups have not been able to confirm the finding.

Despite the strong suspicion that autoantibodies might be important in IDDM, it was not until 1974 that autoantibodies against some component in the islet cell cytoplasm were detected in patients with diabetes mellitus and other autoimmune endocrinopathies⁶. Similar autoantibodies were soon confirmed in other patients and have now been found in over 2,000 diabetic patients who do not have polyendocrine failure (see review in ref. 7).

As with other autoimmune features of IDDM, the exact prevalence of these autoantibodies depends on the duration of disease. Within the first one to two weeks of onset, they will be detectable in up to 85 per cent of diabetic children. This percentage rapidly declines, reaching 25 per cent by two to five years after diagnosis. The autoantibodies are present in 1–2 per cent of normal controls but their prevalence is increased in first-degree relatives of patients with IDDM and in patients with NIDDM. They are invariably of the IgG class, and, in most cases, react with the cytoplasm of all four major types of islet cells — the insulin-secreting β cells, glucagon-secreting α cells, somatostatin-secreting δ cells and pancreatic polypeptide-secreting PP cells. These autoantibodies may not be pathogenic in IDDM as only β cells are destroyed.

More recently, a complement-fixing immunofluorescence assay for detection of anti-islet cell cytoplasm autoantibodies has been used⁸. These complement-fixing antibodies seem to correlate more closely with disease. In addition, in a study currently underway, these antibodies appear to be a predictive marker of impending development of IDDM in the non-diabetic siblings of affected children.

In 1976, using a culture of insulinoma, a new class of islet cell antibodies that reacted with the surface membranes of islet cells was found⁹ and later those observations were extended using dispersed rat



Possible pathogenesis of insulin-dependent diabetes mellitus.

islet cells¹⁰. Like the islet cell cytoplasm autoantibodies, the membrane autoantibodies are most prevalent in the early-onset diabetic. In the presence of complement, cell surface autoantibodies, but not cell cytoplasm autoantibodies, stimulate chromium release from radiolabelled islets and inhibit insulin release, suggesting direct cytotoxicity to islet cells¹¹. Cell surface autoantibodies, however, are also found in the sera of non-diabetic first-degree relatives of patients with IDDM.

The antigens at which the autoantibodies are directed remain to be identified. An immunoprecipitation technique¹² shows that most of the sera which contain them react with a protein of molecular weight 64,000 in ³⁵S-methionine-labelled human islet cell lysates. Hopefully, the determination of the nature of the antigen will provide a clue as to the earliest lesion in IDDM.

Circulating immune complexes have also been identified in patients with IDDM¹³. They are present not only in those patients with recent onset of disease, but also in the late phase, and in patients with NIDDM. Some of these may be immune complexes involving anti-insulin antibodies, but the widespread nature of their occurrence suggests that other antigens must be involved also.

Several animal studies suggest that virus or toxin may be the triggering factor in a genetically susceptible individual. Diabetes-like syndromes can be produced in mice by infection with encephalomyocarditis virus or reovirus^{14,15}. Reovirus also triggers production of autoantibodies that react with antigens on the surface of islet and pituitary cells, as well as antibodies that react with the hormones secreted by these cells. Likewise, insulinitis and diabetes-like syndromes can be produced by injection of low doses of β -cell toxins, such as streptozotocin¹⁴. In a series of elegant studies, Notkins and co-workers have shown that the effect of these environmental factors may be additive, and clearly varies with the genetic background of the animal¹⁵.

In humans, the precise nature of the genetic influence in the pathogenesis of

diabetes mellitus remains unclear as IDDM occurs concordantly in only about 50 per cent of identical twin pairs¹⁶. Alleles of the major histocompatibility system (the HLA system) are clearly associated with the occurrence of IDDM. Both HLA DR3 and DR4 are associated with a three- to fivefold increase in risk for IDDM¹⁷. Although there is no difference in risk between heterozygotes and homozygotes, the risk of disease to the double heterozygote — the individual with DR3/DR4 — increases almost 10-fold, suggesting that the effect of these antigens is dominant but involves independent mechanisms. HLA antigens in linkage disequilibrium with DR3 and DR4, such as B8 and B15, show similar associations. HLA B8/DR3 are associated with a persistence of islet cell surface autoantibodies in patients with IDDM^{7,8} and also appear associated with antibodies to a specific 38,000 molecular weight antigen¹². By contrast, NIDDM is not associated with any specific HLA alloantigens, but is clearly genetically influenced since it occurs in identical twins with almost total concordance¹⁶.

The recognition of the possible important role for autoimmunity in IDDM has led to attempts to suppress immune response. In mouse and rat models of IDDM, immune suppression by irradiation, neonatal thymectomy, anti-lymphocyte serum or bone marrow transplant decreases the incidence of clinical disease¹⁸. At present it is too soon to know whether these techniques will alter the course of the human disease, but they certainly present hope for the first new approach to the therapy of IDDM since the introduction of insulin over 60 years ago. □

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ERRATUM

In *The NK cell: a phagocyte in lymphocyte's clothing?* (298, 511) an error in our labelling of a graph led to the statement that O₂⁻ production by NK cells exceeds that of neutrophils by two orders of magnitude; the actual production rate is about one-tenth that of neutrophils.

ARTICLES

Blue sunlight extinction and scattering by dust in the 60-km altitude atmospheric region

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Twilight data obtained photographically from a stratospheric balloon platform in the autumns of 1980 and 1981 and in the spring of 1982 are presented for blue and red light. They indicate the presence of a light absorbing layer and of a scattering layer in the mesosphere at altitudes near 60 ± 10 km with a low scattering albedo (0.1) at $0.44 \mu\text{m}$ if it is accepted that both the extinction and scattering originate from dust. The optical efficiency of the layer increases more than 10 times when the wavelength of the interacting light changes from 0.65 to $0.44 \mu\text{m}$. At the zenith and near sunset, the natural $0.44\text{-}\mu\text{m}$ extinction optical thickness and the cm^2 column scattering rate due to this layer are found to be 6.6×10^{-2} and $0.18 \text{ MR } \text{\AA}^{-1}$ respectively on 3 May 1982 above the south-west of France.

AEROSOLS have already been optically observed in the upper atmosphere from spacecrafts^{1,2} and rockets³⁻⁵. Twilight observations^{6,7} are also consistent with an upper-atmospheric particulate scattering. Other relevant experimental and theoretical work about upper atmospheric aerosols has been reviewed recently⁸ in a study of their possible continuous and sporadic extraterrestrial sources and fate. On the other hand, it has been known for more than 40 years⁹ that the atmosphere contains a source of optical extinction other than air and ozone in a supposedly transparent spectral region around $0.44 \mu\text{m}$. This extinction has been observed day and night using stars and the Sun as light sources and has caused some concern to those¹⁰⁻¹² interested in the accurate determination of the extraterrestrial solar flux who have attributed the extraextinction to aerosols. It has more recently been attributed^{13,14} entirely to NO_2 but the amount of this atmospheric constituent so deduced disagrees with observations based on the NO_2 extinction of IR radiation¹⁵⁻¹⁷ and based on the differential extinction measurement of blue light¹⁸⁻²³. Both the latter methods yield a smaller nitrogen dioxide abundance, 10–100 times less. Several authors^{20,21,23} have indicated the presence of an unattributed absorption at $0.44 \mu\text{m}$ in the upper stratosphere.

Observation method

The observation method used here has been described previously²⁴ and leads to the determination for various scattering angles, θ , of the radiance of the sunlit atmosphere in units of solar radiance as given by

$$R_\theta/R_\odot = 6.79 \times 10^{-5} [Q_s n \sigma_\theta / 4\pi + n_M \sigma_\theta] \quad (1)$$

where Q_s , σ , ϕ_θ and n are the aerosol scattering efficiency, the geometrical cross-section, the phase function and the number of particles on the line of sight and where n_M and σ_θ are the number of air molecules on the line of sight and the differential air scattering cross-section²⁵.

Two improvements were implemented in the gondola. A neutral density filter identical to the one used to photograph the Sun at elevation angles larger than 5° was remotely moved in front of the cameras to photograph the solar image at zenith angles from 76° to 95° allowing to perform atmospheric absorption measurements. When the Sun was at elevation angles larger than 5° , the lower neutral density screens being removed from the cameras field of view, the gondola was rotated about its

vertical axis by steps of 36° to obtain overlapping pictures of the Earth limb. The spectral bandwidths were $0.06 \mu\text{m}$ and $0.10 \mu\text{m}$ centred at $0.44 \mu\text{m}$ and $0.65 \mu\text{m}$ respectively.

The solar irradiance, I_0 , is attenuated by the atmosphere leading to the measured irradiance

$$I = I_0 e^{-\tau} \quad (2)$$

where the optical thickness

$$\tau = \sum (\sigma_g n_g) + [(Q_s + Q_a) n \sigma] \quad (3)$$

where, σ_g and n_g are the extinction cross-sections of atmospheric gases and their numbers of molecules on the optical path whereas Q_a is the absorption efficiency of the aerosol. Q_s , n and σ have been defined in equation (1). The scattering albedo of the aerosol is

$$\omega = \frac{Q_s}{Q_s + Q_a} \quad (4)$$

and the extinction efficiency of the aerosol is

$$Q_e = Q_s + Q_a \quad (5)$$

Observations

The 1981 balloon flight took place from Aire sur l'Adour in the afternoon of 19 October. During the ascent in the stratosphere and at ceiling altitude, the winds carried the balloon mainly southwards in such a way that observations took place near 43°N and $0^\circ 30'\text{W}$ from 1530 to 1730 h GMT. Radar tracking was performed by the Centre d'Essais des Landes using a transponder carried with the gondola. Temperature and wind and ozone soundings were made, respectively starting at 1059 and 1336 h GMT from $44^\circ 21'\text{N}$ and $1^\circ 14'\text{W}$. From the beginning of the scattering measurements to the end of the absorption measurements the balloon altitude decreased from 34.3 to 33.2 km.

The observed solar irradiances, I , at $0.44 \mu\text{m}$ are plotted against solar zenith angles, χ° , in Fig. 1a as well as their theoretically evaluated evolutions from $\chi = 91^\circ$ towards smaller and larger values of χ . Rayleigh extinction by air has only been taken into account and represents fairly well the change of I from $\chi = 91^\circ$ to 94.3° . A small maximum of excess extinction is observed centred at $\chi = 92.5^\circ$. It corresponds to an optical thickness $\tau \approx 0.06$ which, if attributed to NO_2 , leads to an integrated amount on the optical path of 10^{17} molecules cm^{-2}

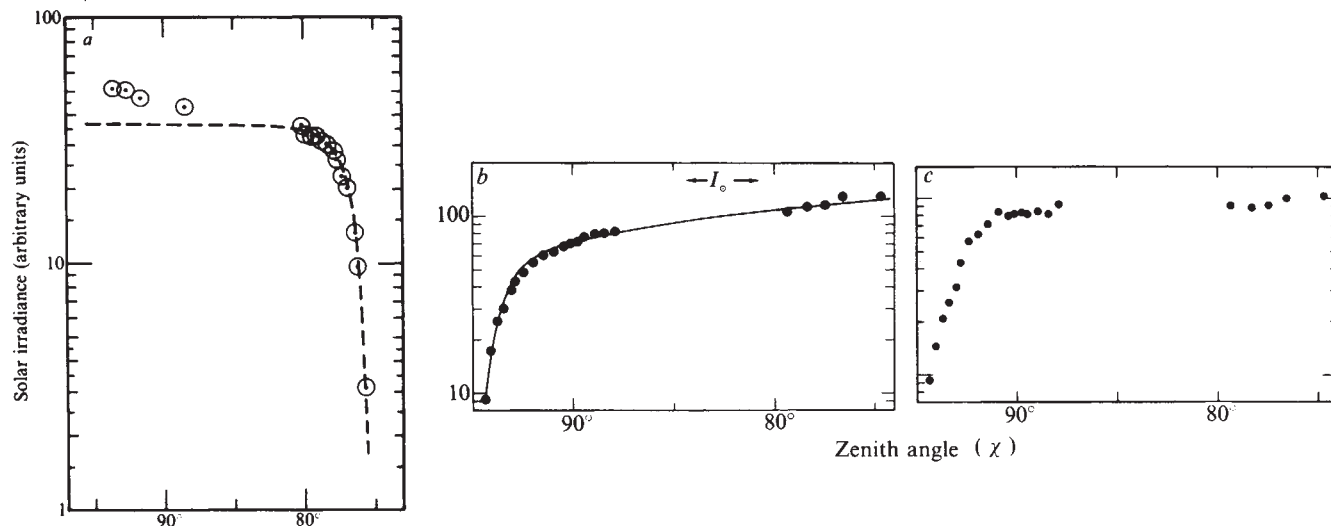


Fig. 1 *a*, Solar irradiances versus solar zenith angles observed on 19 October 1981. The circles represent the measurements while the dashed curve represents the expected variation from Rayleigh extinction, the main expected component at $0.44\ \mu\text{m}$. The observed solar irradiance at $\chi = 91^\circ$ has been taken as a baseline for the computation. *b*, Solar irradiance observed on 3 May 1982 from a flight level of 6.1 mbar at $0.44\ \mu\text{m}$ versus solar zenith angles χ . The values represented by the solid curve have been computed taking the extinction optical thicknesses shown in Fig. 2 and the extraterrestrial solar irradiance indicated by the arrows (I_0). *c*, As *a* but for $0.65\ \mu\text{m}$. The only absorber which can be considered in this case at $\chi = 90^\circ$ is O_3 leading to a radiance reduction of 12%. No excess extinction can be detected with certainty.

at 28 km grazing altitude, a small value, if compared with previous determinations.

We wish to point out the difference between the theoretically expected and the observed evolution of I in the range $90^\circ > \chi > 76^\circ$. An excess of extinction decreasing slowly with increasing solar elevation is observed. This excess has been reported previously as shown in Table 1. Its slow evolution with zenith angle indicates that its cause lies well above flight altitude. No presently known gaseous absorber can explain the observed extinction. At $0.65\ \mu\text{m}$, an optical thickness change of 0.1 can be detected in the measurements from $\chi = 76^\circ$ to 90° corresponding within experimental uncertainties to the expected variation of the ozone extinction. This leads to the first conclusion that a spectrally selective extinction takes place in the upper atmosphere at $0.44\ \mu\text{m}$, of which the origin is up to now unknown. Assuming that the Chapman function is valid for $\chi < 75^\circ$, the total optical thickness at $0.44\ \mu\text{m}$ on 19 October 1981 at $\chi = 90^\circ$ and at 34 km altitude may approach unity.

On 3 May 1982, another evening flight took place from Aire sur l'Adour. The gondola reached a ceiling pressure level equal to 6.1 mbar with a maximum radar measured altitude of 36.6 km. The solar extinction data are shown in Fig. 1*b*. Here again an excess extinction appears at $0.44\ \mu\text{m}$ for solar zenith angles $< 90^\circ$. Figure 1*c* shows the solar extinction curve at $0.65\ \mu\text{m}$. Taking into account an optical thickness of 0.1 in red light due to ozone at a zenith angle of 90° , no additional or excess absorption can be measured reliably.

The solid curve fitting the data on Fig. 1*b* is the result of a computation taking into account the optical thicknesses vari-

ation versus zenith angle shown in Fig. 2 for air (Rayleigh scattering), NO_2 and X, the unknown absorber.

To evaluate the effective altitude of X, the fraction of its maximum optical thickness τ , reached at $\chi = 90^\circ$, has been computed for various values of χ . The result is shown in Fig. 3*a*. The relative value of the extraterrestrial solar radiance indicated in Fig. 1*b* has been chosen to provide the best fit to a theoretical dependence of τ versus χ leading to an effective altitude of $60 \pm 10\ \text{km}$.

The NO_2 optical thickness shown in Fig. 2 associated with a molecular extinction cross-section of $6 \times 10^{-19}\ \text{cm}^2$ leads to a total stratospheric vertical content of NO_2 of 10^{16} molecules cm^{-2} .

The Earth limb radiances observed in the range of zenith angles, χ° , from 86° to 93° are shown in Fig. 4 for two flights: 15 October 1981 at 37 km altitude (only mainly forward scattering is shown at $0.44\ \mu\text{m}$ and at $0.65\ \mu\text{m}$) and 19 October 1981 at 34 km altitude (scattering angles from 14.5° to 166° at $0.44\ \mu\text{m}$ and from 10.5° to 51° at $0.65\ \mu\text{m}$). The accuracy of the absolute values of $R_\theta/R_\odot$ are considered to be $\pm 50\%$ from one camera to the next, mostly due to the uncertainty in the evaluation of the high extinction of the neutral density screens. The accuracy of the relative values on a single camera is $\pm 5\%$. The radiance variation observed compared with χ° on 15 October 1980 and shown here for $\theta = 13^\circ$ is the best illustration

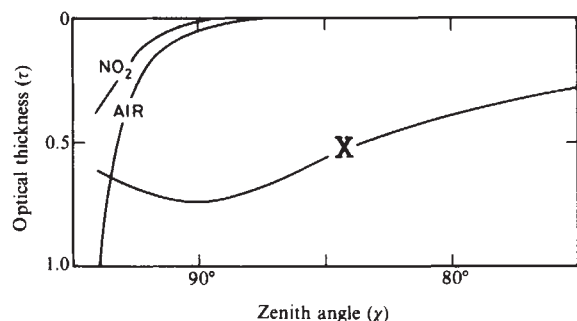


Fig. 2 Optical extinction thicknesses due to air, NO_2 and to the excess absorber, X, necessary to interpret the extinction observations of Fig. 1*b*.

Table 1 List of extinctions in excess of Rayleigh scattering extinction observed at or near $0.44\ \mu\text{m}$ and at solar zenith angles $\chi \approx 90^\circ$ by means of instruments flown on various dates at various altitudes in the stratosphere

Date	Flight altitude (km)	Range of χ°	Wave-length (nm)	$\Delta\tau$	Ref.
9 February 1977	40	75–80*	445	0.18	20
15 October 1980	38	81–84	440	0.28	This work ²⁴
19 October 1981	34.1	76–82	440	0.19	This work
19 October 1981	34.1	82–90	440	0.22	This work
June–July 1973	16.0	90° → "	430	0.57	13

The changes of optical thickness, $\Delta\tau$, are indicated for various ranges of zenith angles. The observations made from Concorde in June–July 1973 lead to an absolute value of τ deduced from absolute solar irradiance data measured at 16 km altitude and at $\chi = 90^\circ$ compared with extraterrestrial solar flux values.

* τ has deduced from a least square fit to the data available in this range of χ° .

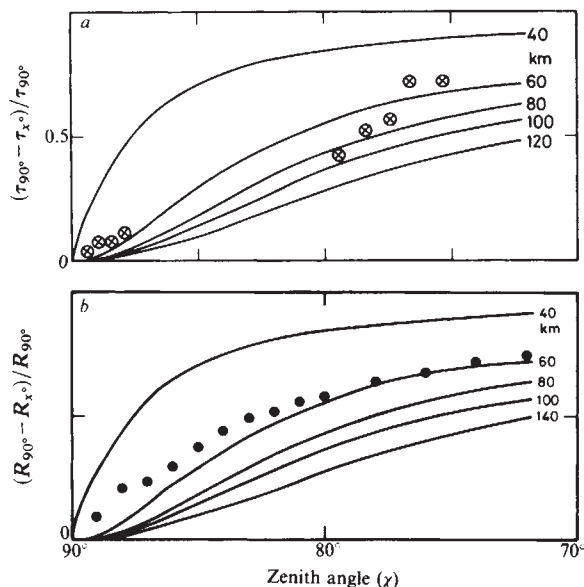


Fig. 3 *a*, Fraction of the excess extinction at $\chi = 90^\circ$ plotted against zenith angles, χ . The computed variation of this quantity versus χ for various effective altitudes (40, 60, 80, 100 and 140 km) of the X absorber. *b*, As *a*, the fraction of the excess radiance observed at $\chi = 90^\circ$ is plotted against χ . The expected variation of this quantity is also shown for various effective altitudes. It indicates an effective altitude of the excess radiance of nearly 60 km.

of the phenomenon that we wish to emphasize. Because at $0.44 \mu\text{m}$, the Rayleigh scattering should dominate the scattering, a theoretical variation of the total number of air molecules on the line of sight versus χ is fitted to the observed radiance curve at $\chi = 90^\circ$. At χ larger and smaller than 90° the observed radiance variation with χ is smaller than the theoretical one represented by the dotted curve. The cause of this difference is considered to be an excess of radiance originating from altitudes larger than the flight altitude. The dotted theoretical curve is shifted downwards to fit the observations at $\chi \approx 92.5^\circ$ where the excess of radiance becomes small compared with the air Rayleigh radiance, because lower and lower altitudes are tangentially observed at progressively larger zenith angles

where the increasing air density must eventually dominate the scattering dependence of zenith distance. It is also observed that the excess radiance decreases slowly above the horizontal line of sight when χ° becomes $< 90^\circ$. The same phenomenon is observed on 19 October 1981 at $0.44 \mu\text{m}$ and at all scattering angles. In this case, however, the excess appears relatively smaller since the flight altitude was lower leading to a larger Rayleigh scattering due to air. A decrease of the blue and red colour ratio occurring near 1° solar depression angle, δ° , has been observed in twilight studies⁶ as well as an excess of blue light at $\delta = 0^\circ$ which was attributed to multiple scattering. In the case of our observations, however, the air observed at $\chi \leq 90^\circ$ is theoretically optically thin and multiple scattering does not explain the radiance excess, relative to pure air, linked with the anomalous radiance variation with zenith angle.

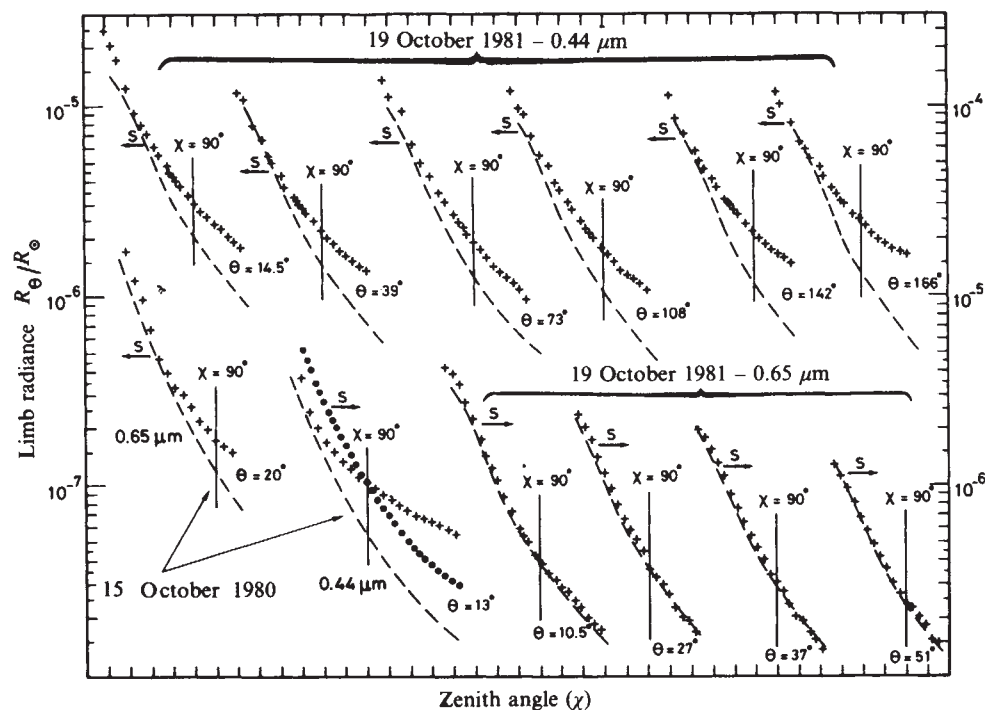
At $0.65 \mu\text{m}$, a small excess of radiance is observed at $\theta = 20^\circ$ on 15 October 1980. It is barely detectable at $\theta = 10.5^\circ$ on 19 October 1981 and it cannot be detected at larger scattering angles, being probably too small relative to Rayleigh scattering at this low flight level.

For the 3 May, 1982 flight, one of the cameras was fitted with a blue filter-equipped wide angle lens ($f = 50 \text{ mm}$) instead of the lens normally used ($f = 80 \text{ mm}$). No neutral density screen was placed in front of this camera so that the sky could be viewed over a wider range of zenith angles. The calibration of this camera was performed by comparing film optical densities of identical features simultaneously photographed by means of another blue filter, neutral density screen equipped, camera.

The limb radiances observed at two flight levels, one during the balloon ascent and the other one at ceiling altitude, are plotted in Fig. 5 against zenith angles. The variations of the number of air molecules viewed on the optical path versus zenith angles are also represented and fitted to the observations at large zenith angles ($> 90^\circ$) where pure air Rayleigh scattering must dominate.

As for the extinction in Fig. 3a, the fraction of the total excess radiance at $\chi = 90^\circ$ has been plotted against zenith angles in Fig. 3b. Here again the measurements show an effective altitude for the excess radiance near 60 km. The radiance was also observed at $0.65 \mu\text{m}$ for two flight levels as it was done in Fig. 5 for $0.44 \mu\text{m}$. The excess radiance determined in red light is 11 times smaller than in blue light. By spinning the gondola about its vertical axis by steps of 36° , the data necessary for

Fig. 4 Earth limb radiance $R_\theta/R_\odot$ in units of solar radiance observed on 15 October 1980, at 37 km altitude and on 19 October 1981 at 34 km altitude versus zenith angles χ° . The observations are represented by crosses. The dashed and dotted (see text) curves represent total numbers of air molecules in arbitrary units which can be seen on the line of sight versus zenith angles χ° . The arrows marked 's' indicate the ordinate scale relevant for each graph. The scattering angles θ° , the wavelengths, $0.65 \mu\text{m}$ or $0.44 \mu\text{m}$ and the dates are also indicated. Average solar zenith angles were 78° on 19 October 1981 and 80° on 15 October 1980. For each case a vertical line indicates the $\chi = 90^\circ$ position on the abscissa where an angular difference of 1° separates two ticks, zenith angles increase towards the left-hand side of the figure.



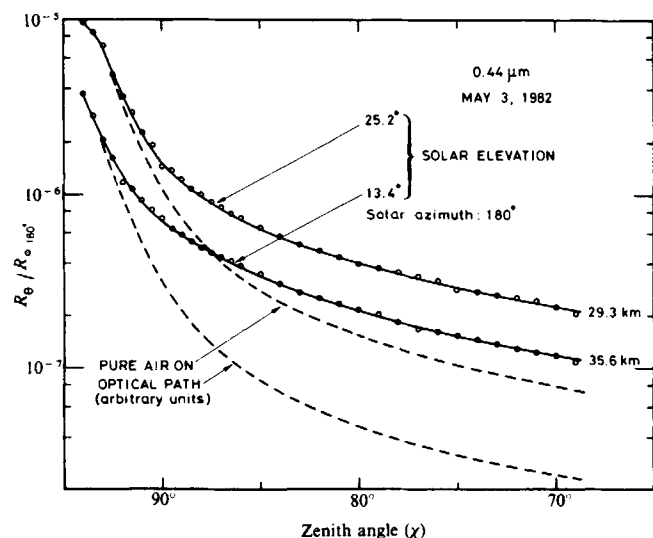


Fig. 5 Atmospheric radiance plotted against zenith angle at $0.44 \mu\text{m}$ from two flight levels on 3 May 1982. The pure air expected radiance is represented by the dashed curves.

the determination of the phase function of the excess radiance have been obtained.

The phase functions are shown in Fig. 6 for three zenith angles in the case of the excess radiance. The values for air are those measured at $\chi = 90^\circ$. However, in the latter case the angular variation reflects the phase function at zenith angles close to 93.5° , corresponding to grazing altitudes near 20 km. At these low altitudes the low stratospheric aerosols and perhaps the Earth sphericity induce illumination asymmetries when the solar azimuth changes from 36° to 180° . This most probably explains the deformation of the measured air phase function shown in Fig. 6 for air. At smaller zenith angles the air Rayleigh scattering becomes quickly very small compared with the excess radiance for which it becomes only a correction factor. The phase function observed at $\chi = 80^\circ$ is for this reason used to deduce its characteristics.

The value of $Q_s n \sigma$ has been evaluated from these experimental data by means of the relationship

$$Q_s n \sigma = 1.85 \times 10^5 \sum_{0^\circ}^{180^\circ} (R_\theta / R_\theta(90^\circ)) (\sin \theta \sin \Delta \theta / 2) \quad (6)$$

by zonal summation every 10° between 0° and 180° . The extinction due to scattering is found to be 2.6×10^{-2} which, according to Fig. 3b, leads, at $\chi = 90^\circ$, to $Q_s n \sigma = 6.1 \times 10^{-2}$. On the other hand, the asymmetry parameter, $\langle \cos \alpha \rangle$, is found to be 0.06.

Discussion

The extinction and the excess of radiance have been shown to originate from an altitude near 60 km, in the mesosphere. They both also exhibit a large enhancement when the wavelength changes from 0.65 to $0.44 \mu\text{m}$.

Received 5 April; accepted 18 June 1982.

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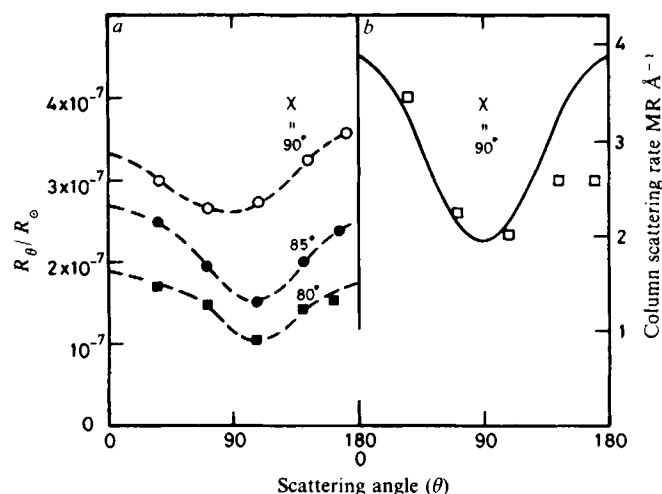


Fig. 6 Atmospheric excess radiance (a) observed at various zenith angles plotted against scattering angles θ° . b, The pure air radiance phase function computed for the flight level (at 6.1 mbar) from the air differential cross-section²⁵. Solar elevation is taken as $9^\circ \pm 1.5^\circ$.

For an effective height of 60 km and an observation altitude of 35 km the optical depth factor increases 11.4 times from $\chi = 0^\circ$ to 90° . The excess extinction observed leads, according to Fig. 3, to a zenithal optical depth of 6.6×10^{-2} of which 5.4×10^{-3} is due to scattering by the dust layer. If it is accepted that the dust is responsible for both extinction and scattering in the 60-km layer, the dust scattering albedo is 0.08. This low value is compatible with very small particles exhibiting an almost symmetrical scattering phase function. One way to explain the large scattering efficiency increase when the wavelength changes from 0.65 to $0.44 \mu\text{m}$ is to consider a colour-dependent complex index of refraction in this range with a non-negligible imaginary part of the index²⁶. The dust would thus be made out of brownish matter. The complexity of the upper atmospheric dust probably having an extraterrestrial origin has already been pointed out²⁷.

Conclusion

The demonstration that a dust layer is present in the Earth's atmosphere at 60 km altitude would have a purely academic interest if it were not for its association with a non-negligible sunlight extinction and absorption *in situ* which makes it important for the Earth radiation balance, for the stratospheric and mesospheric photochemistry and energy budget and for the measurements of stratospheric trace species in blue light. These aspects justify the continued optical investigation of the layer from balloon gondola and from other platforms, and the use of other methods of investigation.

We thank the French Centre National d'Etudes Spatiales for the balloon operations and the Etablissement d'Etudes et de Recherches Météorologiques for atmospheric soundings.

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Basalt genesis and mantle structure studied through Th-isotopic geochemistry

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The relative importance of the degree of partial melting and of the composition of the source, is investigated for basalts of various origins on the basis of observed Th isotopic variations. If the oceanic island basalts (OIB) and the mid-oceanic ridge basalts (MORB) are not mutually contaminated, the Th isotopic tracers show that the difference between MORB and OIB is due to a difference in the composition of their sources rather than in the degree of partial melting. The characteristics of the island arc volcanics are completely different from those for the oceanic basalts, thereby implying a difference in both their origin and their geochemical process of petrogenesis.

THE determination of the sources and the magmatic processes for basalt genesis is a key problem in petrology^{1,2}. The relative importance of these two factors in determining the different types of volcanism is also crucial. The current (popular or accepted) ideas for the three major types of volcanism can be summarized as follows. The mid-oceanic ridge basalts (MORB) would result from a large degree of partial melting whereas oceanic island basalts (OIB) mainly alkalic or of alkalic affinity) correspond to a small degree of partial melting. The difference in composition of the initial material, proposed on a geochemical basis, especially from isotope studies, seems to be secondary relative to effect of the degree of partial melting, at least for the major elements. For trace elements, especially the incompatible elements, the effect of a different source or of a different degree of partial melting would be about the same.

However, the difference between MORB and island arc basalts (or andesites) would mainly result from a major difference in the composition of the sources, the MORB originating from a depleted mantle, mainly peridotitic, whereas the island arc basalts originating either by the melting of a basic downgoing slab (in the eclogite or amphibole facies) with possible mixing with sediments of continental crust, or from the melting of an hydrated peridotitic wedge.

We attempt to show here how the use of the (²³⁰Th/²³²Th) isotopic ratios measured in volcanic rocks can help in clarifying such problems and in deciphering whether the nature of the source or the processes of formation are at the origin of the differences observed among basalts. (In what follows the ratios in parentheses are activity ratios and those between square brackets are atomic ratios.)

(²³⁰Th/²³²Th)–⁸⁷Sr/⁸⁶Sr correlation in recent volcanic rocks

The measurement of (²³⁰Th/²³²Th) ratios for recent lavas from several oceanic areas has shown the existence of an anticorrelation relative to their [⁸⁷Sr/⁸⁶Sr] ratios (Fig. 1)³. The most likely interpretation for this anticorrelation is that the source regions for the basalt volcanism of these various zones are in radioactive equilibrium, and that the (²³⁰Th/²³²Th) ratios of the lavas are representative of the (²³⁸U/²³²Th)_S ratios of the mantle source, which implies a negligible time of stay at depth and of transfer of these magmas to the surface (a few thousand years at the most) relative to the ²³⁰Th half life (75,200 yr). The (²³⁰Th/²³²Th)–[⁸⁷Sr/⁸⁶Sr] anticorrelation is then indicative of a correlation between [Th/U] and [Rb/Sr] ratios of the source regions, hence of a consistency of the Th–U, Rb–Sr and Nd–Sm chemical fractionations. A low [⁸⁷Sr/⁸⁶Sr] ratio corresponds to a mantle source with time-integrated low [Rb/Sr] and [Th/U] ratios, and thus high (²³⁰Th/²³²Th) ratio. The 'bulk Earth' value of [Th/U] ratio (~3.7) can be derived from Pb isotopic studies⁴.

This value corresponds on the correlation diagram to a [⁸⁷Sr/⁸⁶Sr] value of ~0.7048, which is precisely the 'bulk Earth' Sr isotopic ratio obtained through the Sm–Nd correlation. The consistency between these different isotopic tracers is thus confirmed. We must then admit that, in the mantle, there are depleted zones for which the [Th/U] ratio is low (high (²³⁰Th/²³²Th) and low [⁸⁷Sr/⁸⁶Sr] ratios): they are the source regions of most of the oceanic volcanism, with a minimum [Th/U] value of around 2.3. The complement of these depleted sources is probably the continental crust which should have a [Th/U] ratio higher than 3.7.

A correlation between isotopic ratios and trace elements ratios had already been suggested previously for the MORB^{5,6}. This correlation was nonlinear. When using the Th isotopes, we do not observe any curvature in the correlation which suggests that this curvature results from a chemical fractionation in the trace element ratios created by partial melting (see Fig. 1, inset).

The comparison of the actual (²³⁸U/²³²Th) ratios of the lavas and of their (²³⁰Th/²³²Th) ratios as representative of the (²³⁸U/²³²Th) ratios of the source regions, yields a simple and unique way of determining the U–Th fractionations due to the whole magmatic processes. Because the differentiation phenomena through fractional crystallization have little effect on the [Th/U] ratio, at least for oceanic volcanism, most of the Th–U fractionation can be ascribed to partial melting³. Figure 2 illustrates this process in a (²³⁰Th/²³²Th)–(²³⁸U/²³²Th) isochron diagram⁷. The point S represents the mantle source in radioactive equilibrium. Through partial melting a magma M is produced with an identical (²³⁰Th/²³²Th) ratio but a different (²³⁸U/²³²Th). The fractionation produced may be represented by a factor:

$$k = (^{238}\text{U}/^{232}\text{Th})_{\text{M}} / (^{238}\text{U}/^{232}\text{Th})_{\text{S}}$$

where M and S stand for the magma and the mantle source respectively. Since

$$(^{238}\text{U}/^{232}\text{Th})_{\text{S}} = (^{230}\text{Th}/^{232}\text{Th})_{\text{S}} = (^{230}\text{Th}/^{232}\text{Th})_{\text{M}} \\ k = (^{238}\text{U}/^{230}\text{Th})_{\text{M}}$$

Obviously, if the time of stay at depth and of transfer of the magma to the surface is significant relative to the ²³⁰Th half life then the point representative of the magma shifts along a vertical line, downwards if this point is to the left of the equiline ((²³⁰Th/²³⁸U) > 1) or upwards if the point is to its right ((²³⁰Th/²³⁸U) < 1) (Fig. 2), considering that the differentiation has no effect on the (²³⁸U/²³²Th) ratio of the magma. In this case, to determine the fractionation due to partial melting we must consider the (²³⁰Th/²³²Th)₀ initial ratio of the magma at the time of its formation. If the time of stay at depth of the

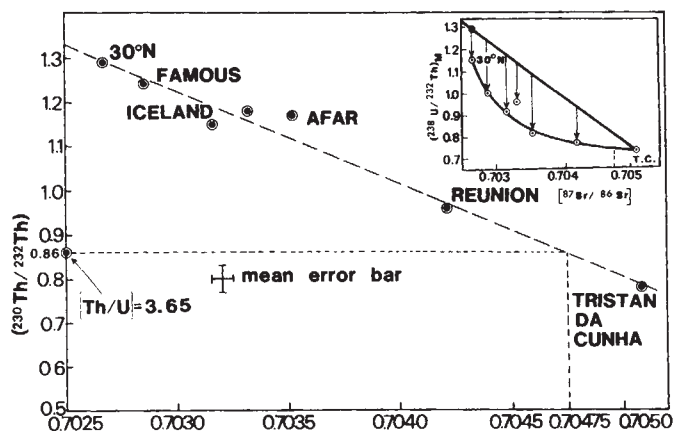


Fig. 1 Correlation diagram between $(^{230}\text{Th}/^{232}\text{Th})$ and $^{87}\text{Sr}/^{86}\text{Sr}$ ratios (from ref. 3). Inset, correlation diagram between $(^{238}\text{U}/^{232}\text{Th})_{\text{M}}$ ratios of the magmas and $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratios (note the curvature of this correlation).

magma is important, its representative point on the diagram is shifted away from the $(^{230}\text{Th}/^{232}\text{Th})$ - $[^{87}\text{Sr}/^{86}\text{Sr}]$ correlation line because of the change in the $(^{230}\text{Th}/^{232}\text{Th})$ ratio due to radioactive decay. In this hypothesis, which may be verified through the study of the $(^{230}\text{Th}/^{232}\text{Th})_0$ initial ratios evolution of the lavas throughout the history of the volcanic region, the measurement of the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio yields the $(^{230}\text{Th}/^{232}\text{Th})_0$ ratio of the initial magma through the Th-Sr isotopic correlation, hence, the fractionation parameter k for partial melting.

Characteristics of partial melting

Figure 3, in which data for recent lavas from various regions that have already been studied in detail^{3,8-12} are plotted, clearly shows that most of the regions studied plot to the left of the $(^{230}\text{Th}/^{232}\text{Th})$ - $(^{238}\text{U}/^{232}\text{Th})$ diagram. This is the case for oceanic lavas such as those from the FAMOUS and 30°N zones on the mid-Atlantic ridge, for lavas from Afar, from oceanic islands such as Iceland, Hawaii, Faial (Azores) and La Réunion, from Etna and Stromboli¹³ and those from the continental alkali volcanism of the Chaîne des Puys (Massif Central, France).

However, some lavas plot to the right of the equiline. These all come from subduction zone volcanism such as Costa Rica⁸ and Japan (unpublished data). This type of volcanism, thus, seems quite distinct from the general case for which $(^{230}\text{Th}/^{238}\text{U})$ is >1 .

We will now consider the case of the volcanism in oceanic regions and that in subduction zones.

Oceanic basalts

The fact that all recent oceanic lavas plot to the left of the equiline has a fundamental implication about the partial melting. The latter fractionates the $[\text{Th}/\text{U}]$ ratio (whereas crystal fractionation does not) and would produce a magma with a $[\text{Th}/\text{U}]$ ratio larger than that for the mantle source. In other words, the melt is preferentially enriched in Th relative to U ($D_{\text{Th}} < D_{\text{U}}$). Indeed, these studies demonstrate that most of the oceanic lavas originate from a mantle source with $[\text{Th}/\text{U}]$ ratios lower than the primitive value of 3.7 (ref. 3), thus, from a source which has already been depleted through previous partial melting events. This is in full agreement with the conclusions from Pb isotopic studies⁴. Thus, the oceanic tholeiites with the most depleted character in Pb isotopes (as in Sr or Nd) have the highest $(^{230}\text{Th}/^{232}\text{Th})$ ratio values hence the lowest $[\text{Th}/\text{U}]$ ratios for their source regions (Fig. 3).

Such a result shows that despite their highly magmaphile character, $[\text{Th}/\text{U}]$ ratios do fractionate during partial melting and therefore, using the $[\text{Th}/\text{U}]$ ratio measured in lavas to infer $[\text{Th}/\text{U}]$ mantle geochemistry and heterogeneities, is a risky exercise. This applies to Th and U, which are among the most magmaphile elements, but also to other elements such as rare

earth elements, Ta, Hf and, of course, to Tb, Sr, Sm, Nd (see refs 14, 15). A most important factor is the degree of partial melting which can be estimated through the parameter (k) previously defined as the Th-U fractionation factor between the mantle source and the magma. The larger this fractionation, the lower the degree of partial melting considering that the partition coefficients D_{U} and D_{Th} remain almost constant. The determination of the latter would allow a calculation of the degree of partial melting. This assumption that the partition coefficients D_{U} and D_{Th} remain constant might be an oversimplification. Indeed, we do not know which minerals are involved in Th-U fractionation during partial melting. Tatsumoto⁴ assumed that, among the main minerals of peridotite, clinopyroxene is the major phase involved in this fractionation. But, he emphasized that the experimentally determined clinopyroxene-melt partition coefficients indicate a Th-U fractionation in contradiction with the Pb isotopic data (that is a melt with a Th/U ratio lower than that of the mantle). He concluded that either the experimental clinopyroxene-melt partition coefficients were not applicable to natural conditions during partial melting, or that other minerals such as phlogopite, amphibole, (apatite) were involved in the partitioning between U and Th.

Nevertheless, it is quite obvious that the percentage of melt is rather variable for tholeiites from oceanic ridges or rift zones (for example ~ 0.9 at 30°N of the mid-Atlantic ridge, $k \sim 0.8$ for the FAMOUS zone and Iceland at 37°N of the mid-Atlantic ridge and $k \sim 0.7$ for a lava from Afar). In an environment of spreading centre there seems to be a relationship between the degree of alkalinity and the degree of partial melting as determined through the parameter k . Indeed, the basalts from Afar are more alkalic than those from the FAMOUS zone, the latter being themselves more alkalic than those from 30°N.

This relationship between the degree of alkalinity and the degree of partial melting determined through the parameter k , seems still to be valid (Fig. 3) for oceanic islands and even continental volcanism (Chaîne des Puys). For example, $k \approx 0.9$ for the tholeiites from Kilauea (Hawaii), $k \approx 0.8$ for the transitional tholeiites from La Réunion, $k \approx 0.7$ for the alkali basalts from the Grande Comore island (Indian Ocean) as well as for those from the Chaîne des Puys (Massif Central, France). Note that the k coefficients (0.9–0.7) appear to be similar to those determined for the MORB, whereas the $(^{230}\text{Th}/^{232}\text{Th})$ ratios are clearly lower and more variable, indicating different, less depleted mantle sources. The difference in the degree of melting does not seem to be the primary cause of the chemical difference between MORB and OIB. The difference between the source regions and their more or less depleted character thus appear more important than the actual percentage of partial melting.

If the OIB were actually derived from a lower degree of partial melting of mantle material than MORB, then the partition coefficients D_{U} and D_{Th} must be different in these two cases and would probably be related to two mantle sources of different chemical and mineralogical composition. As a corollary to what we know about Sr and Pb isotopic ratios, the two

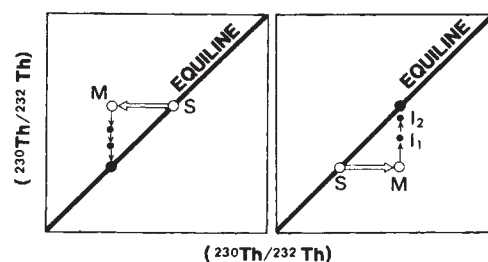


Fig. 2 Isochron diagram showing the geochemical behaviour of the ^{230}Th - ^{238}U system during partial melting and fractional crystallization (see refs 3, 11 for general explanation). S, magma source; M, primary magma; $I_1, I_2, \dots$ different lavas erupted at different times. White arrows represent partial melting, black arrows represent radioactive decay.

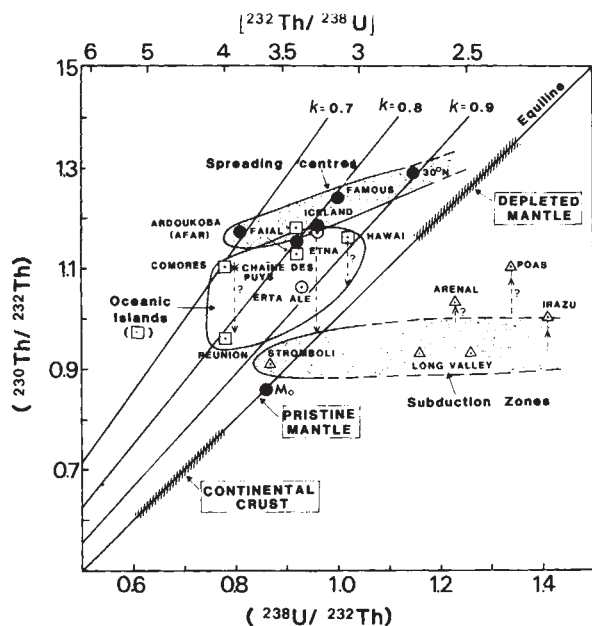


Fig. 3 Isochron diagram showing the different magmas of various sources. Data are from refs 3, 9–13, 16 and Condomines *et al.* unpublished results. The lines corresponding to different fractionation coefficients k are indicated (see text). The upper horizontal axis is calibrated in $[^{232}\text{Th}/^{238}\text{U}]$ atomic ratios. The dashed arrows denote long transfer times of the magmas and the supposed positions of the primary magmas are then indicated (Etna, Chaîne des Puys, Hawaii).

domains representative of the MORB and OIB are not completely disconnected and a gradual transition does exist. Is the latter either a superficial translation of a real chemical zoning in the mantle or, conversely, an indication of mixing and contamination phenomena during the genesis and the transfer of magmas?

In the latter hypothesis, the position of the magma in the isochron diagram is no longer related to the degree of partial melting. Let us consider, for example, the following simple model⁵: an homogeneous lower mantle with a 'primitive' $[\text{Th}/\text{U}]$ ratio of 3.7 and a depleted upper mantle with a $[\text{Th}/\text{U}]$ ratio of ~ 2.3 (this is the minimum value obtained by extrapolating the oceanic trend to the equiline). The partial melting of 'blobs' from the lower mantle would yield the OIB, the partial melting in the upper mantle producing the 'normal' MORB. The mixing of these two magma types during the ascent of 'blobs' could explain the continuity of the two domains. There would be MORB more or less contaminated by the OIB magma and the reverse for OIB. In that case, the extreme values of $(^{230}\text{Th}/^{232}\text{Th})$ ratios (and Pb, Sr, Nd isotopic ratios) would be closest to the real values of these two sources. To explain the scatter of the points in the isochron diagram, each of these two mantle sources must undergo different degrees of melting (Fig. 4). It may be that the truth is in between these two extreme models (the latter simple mixing model and the model with different mantle sources for each volcanic region). The mixing model has been discussed already in the case of Iceland⁹.

It is not possible to make a definitive choice between these two models, but the ^{230}Th – ^{238}U disequilibrium data emphasize source differences among oceanic basalts. These differences might be related to chemical and mineralogical differences in the mantle composition, which would play an important part as well as the actual degree of melting, in determining the composition of the primary magmas.

Subduction zones

So far, there are only a few data available for the volcanism at subduction zones. However, a previous study of Costa Rica⁸ showed the presence of both ancient (Izuru) and recent lavas (Poas, Arenal) for which the representative points plot to the

right of the equiline in the isochron diagram (Fig. 3). This unusual behaviour is also observed for two rhyolites from the Long Valley (Sierra Nevada)¹⁶ and for lavas from the Marianas¹⁷. There are also some lavas which plot to the left of the equiline⁸ or very close to the latter (Stromboli), but it seems significant that all the lavas with $(^{230}\text{Th}/^{232}\text{Th}) < 1$ come from volcanoes in subduction zone. If we admit that the partial melting fractionates the $[\text{Th}/\text{U}]$ ratio in the same manner as in oceanic basalts, then we have to find a secondary phenomenon to explain the particular position of subduction zone magmas. The arc volcanism is most probably at the origin of continental crust extraction responsible for the progressive depletion of the residual upper mantle; thus, the partial melting probably affects the $[\text{Th}/\text{U}]$ ratio in the usual way.

Another important point brought up by the magmatic evolution study of the Izuru volcano, is the variability of the $[\text{Th}/\text{U}]$ ratios of the lavas throughout the history of the volcano⁸. This is an important difference compared with the other volcanic regions studied (FAMOUS zone, Iceland, Hawaii, Etna) for which the $[\text{Th}/\text{U}]$ ratios do not vary very much. This $[\text{Th}/\text{U}]$ ratio variation for lavas from Izuru has been ascribed to a dragging of U through gas transfer during the ascent of the magma from its reservoir to the surface whereas the magma in the reservoir keeps evolving with a constant $[\text{Th}/\text{U}]$ ratio, thereby yielding an almost linear variation of the $(^{230}\text{Th}/^{232}\text{Th})_0$ initial ratios of the lavas relative to $e^{\lambda t}$ (ref. 8). If this hypothesis were correct it would demonstrate the important role of fluids in this type of volcanism and that U is far more affected than Th by the gas transfers. Would not this role of fluids, evident during the magmatic evolution, also be crucial for explaining the particular position of the magmas from subduction zones to the right of the equiline? The disequilibrium illustrated by the position of a magma to the right of the equiline may either be acquired at the time of its formation through partial melting or be the result of a source already in radioactive disequilibrium and having a similar position in the diagram.

Among the models proposed for the genesis of magmas from subduction zones, one does not seem to be compatible with the disequilibrium data: that of simple melting of the subduction oceanic crust. Indeed, oceanic crust has a quite low $[\text{Th}/\text{U}]$ ratio and hence high $(^{230}\text{Th}/^{232}\text{Th}) \geq 1.10$, after the resetting of radioactive equilibrium, inasmuch as an oceanic crust enriched in U by seawater should present even higher $(^{230}\text{Th}/^{232}\text{Th})$ ratios. Yet, most of the magmas from the subduction zones studied systematically have lower $(^{230}\text{Th}/^{232}\text{Th})$ ratios than those for oceanic tholeiites. If the oceanic crust is involved in the partial melting process there must be a mixing of the latter with another component. This other component may either be sediments dragged during the subduction, or the continental crust. Some detritic sediments (for example, clays) may have high $[\text{Th}/\text{U}]$ ratios, hence rather low $(^{230}\text{Th}/^{232}\text{Th})$ ratios when they reach radioactive equilibrium. Therefore, mixing with the oceanic crust at the time of partial melting may decrease the $(^{230}\text{Th}/^{232}\text{Th})$ ratios of the latter. Besides, the melt

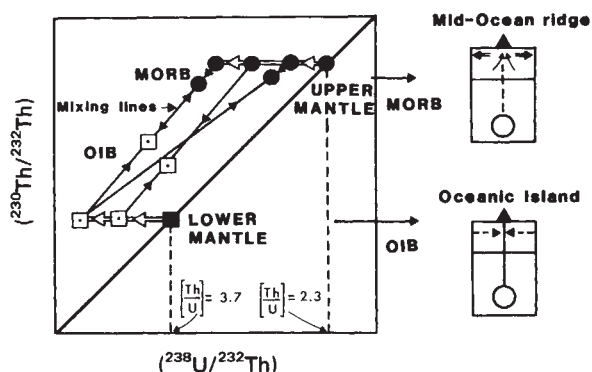


Fig. 4 Isochron diagram showing the possible genesis of MORB and OIB by cross-contamination.

must be preferentially enriched in U relative to Th. The uranium from the sediments could have been brought into the magma by the fluids during partial melting. The contamination by the continental crust (higher [Th/U] ratios) of a magma originating through the melting of the oceanic crust and the participation of fluids from the continental crust is yet another hypothesis which is compatible with the disequilibrium data for explaining the enrichment of the magma in uranium.

We may propose a simple model to account for these remarks: (1) A first melting stage at ridges with creation of MORB; after the spreading, the radioactive decay occurs and the oceanic crust 'falls down' onto the equiline but in a position corresponding to a much less depleted source. (2) A second stage of mixing with sediments and a partial melting in a wet medium which yield the expected result, or interaction of the magma with continental crust and selective contamination in U by continental fluids.

Another model for the genesis of the magmas in subduction zones considers a melting of the mantle 'wedge' right above the subducting plate. This part of the mantle would have been previously enriched in some elements through dehydration of the subducting oceanic crust and through transfer by fluids of the most soluble elements such as Rb or U (mantle metasomatism). This phenomenon could provide an explanation for the

presence of sources in disequilibrium to the right of the equiline. Note that if this model of metasomatism is correct, this phenomenon must have occurred a short time before or during partial melting (the metasomatic fluid could be the cause of partial melting), before the source returned to a state of radioactive equilibrium, which could be indicated by high ($^{230}\text{Th}/^{232}\text{Th}$) ratios that most of the available data do not show.

Considering the limited available ($^{230}\text{Th}/^{232}\text{Th}$) disequilibrium data for the volcanism in subduction zones a definite choice among the above models would be premature. However, fluids would really have an important role either during the partial melting process or after (uranium enrichment of the magma) or even before the melting (uranium enrichment of the source).

A more extensive study of volcanoes from subduction zones (especially from intra-oceanic arcs) would allow confirmation of the disequilibrium position of their magmas to the right of the equiline in the isochrone diagram. In such an instance, it would set an important characteristic for this type of volcanism and yield a method to study its origin.

We thank B. Dupré for an important comment, also R. K. O'Nions and A. Zindler for helpful comments. The manuscript has been prepared by Claude Mercier-Ronnat. This is IPGP contribution NS/599.

Received 30 March; accepted 14 July 1982.

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A study of Si,Al ordering in thallium zeolite-A by powder neutron diffraction

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The cubic structure of thallium zeolite-A has been studied by high resolution powder neutron diffraction. Refinements in which Si and Al are strictly alternating within the aluminosilicate framework (space group Fm3c) are better than those in which each Si is linked, by oxygen bridges, to three Al and one Si (space group Pn3n). This result is in accord with recent ^{29}Si (magic-angle spinning) NMR measurements on zeolite ZK-4 but at variance with previous interpretations of NMR measurements on the sodium form of zeolite-A. Our conclusions reaffirm earlier X-ray results.

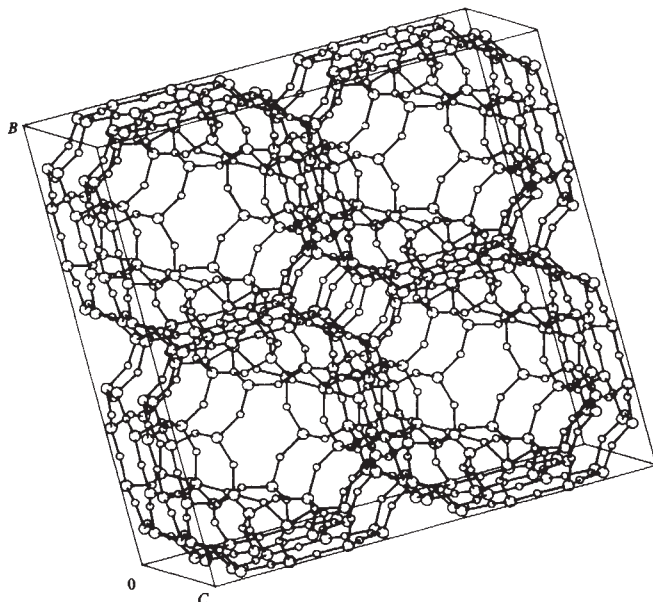
THE growing importance of zeolite materials in catalytic reactions has led to a revival of interest in their microstructure and in particular the details of their Si,Al distributions. Until recently, zeolite-A was regarded as the most definitively characterized member of the zeolite family, with the very simplicity of its structure adding to the assurance that the description was correct. The basic framework consists of silicon and aluminium atoms tetrahedrally coordinated by oxygen (Fig. 1), but the exact ordering of Si and Al has remained the subject of debate. According to Loewenstein's rules¹, a tetrahedrally coordinated aluminium atom should not be linked through oxygen to another aluminium with the same coordination geometry. Although there are exceptions to the rules^{2,3}, they nevertheless provide a good basis for describing aluminosilicate frameworks.

Strict alternation of the Si and Al, in obedience of Loewenstein's rules, leads to a cubic cell, $a \sim 24.6 \text{ \AA}$, and the space group Fm3c (refs 4, 5). In detailed single crystal X-ray studies on sodium zeolite-A⁶ and three samples of dehydrated, potassium zeolite-A⁷, Pluth and Smith obtained excellent refinements in Fm3c, although a few weak reflections violating the c -glide were observed in each case; this was also noted by Thoeni in work on hydrated samples of thallium, calcium and silver zeolite-A⁸.

The apparent violations of the c -glide condition in some of the zeolite-A data suggest the possibility that the space group Fm3c may be incorrect, and recent observations using electron diffraction, neutron diffraction, and high resolution, magic-angle spinning ^{29}Si NMR give cause for further scepticism^{9–14}.

Table 1 Final atomic coordinates for Tl zeolite-A in space group Fm3c

Atom	Position	x	y	z	Occupancy	B(Å ²)
Si	96(i)	0.0	0.0906(5)	0.1826(5)	1.0	0.11(11)
Al	96(i)	0.0	0.1852(5)	0.0884(6)	1.0	0.11(11)
O(1)	96(i)	0.0	0.1069(2)	0.2468(4)	1.0	0.68(7)
O(2)	96(i)	0.0	0.1486(3)	0.1503(3)	1.0	0.68(7)
O(3)	192(j)	0.0532(2)	0.0589(2)	0.1676(1)	2.0	0.68(7)
Tl(1)	64(g)	0.1301(3)	0.1301(3)	0.1301(3)	0.330(10)	3.10(45)
Tl(2)	96(i)	0.0	0.2272(3)	0.2273(3)	0.222(5)	3.10(45)
Tl(3)	64(g)	0.0485(9)	0.0485(9)	0.0485(9)	0.053(4)	3.10(45)
Na(1)	64(g)	0.1025(9)	0.1025(9)	0.1025(9)	0.307(26)	5.33(153)

**Fig. 1** The cubic ($a \sim 12.3$ Å) sub-cell of zeolite-A; Si(Al) and oxygen atoms are shown as large and small spheres, respectively.

The electron diffraction patterns of sodium zeolite-A contradict the space group Fm3c owing to the appearance of hhl reflections with $h+l=2n$ (ref. 15), and neutron diffraction measurements on dehydrated samples of the same material show a pronounced rhombohedral distortion. The NMR studies reveal a single peak with a chemical shift (89.7 ± 0.5 p.p.m.) in the range expected for Si surrounded by three aluminium atoms and one silicon^{12,13}. This 3:1 ordering scheme requires similar ordering for Al, in contradiction of Loewenstein's rule. A rhombohedral structural model that incorporates the 3:1 Si,Al ordering scheme has been advanced by Bursill *et al.*⁹.

In an attempt to resolve the controversy surrounding the Si, Al ordering in zeolite-A, we have carried out a powder neutron diffraction study on one of the rhombohedrally distorted zeolite-A samples, having exchanged sodium for thallium. The greater neutron scattering length of Tl, compared with Na, affords the advantage of locating the exchangeable cations with greater precision.

Table 2 Bond lengths for Tl zeolite-A (Å)

Si-O(1)	1.608(14)	Al-O(1)	1.702(14)
Si-O(2)	1.628(14)	Al-O(2)	1.755(14)
2 × Si-O(3)	1.560(13)	2 × Al-O(3)	1.726(13)
Average Si-O	1.599	Average Al-O	1.728

Experimental work

The sample used in this work was prepared from the Na zeolite-A sample, Si/Al = 1.10, studied in ref. 11. Thallium was incorporated by ion-exchange at 85 °C using a saturated thallium(I) nitrate solution. The solution was changed after 24 h and the procedure repeated. Examination of the product in a Jeol 100CX TEMSCAN analytical electron microscope showed that the sodium had not been fully exchanged.

Neutron diffraction data were collected on the high resolution, powder diffractometer, D1A, at the high flux beam reactor in Grenoble, as described in ref. 14. The data were measured at room temperature in the 2θ range from 6° to 140°, with a mean neutron wavelength of 2.98 Å, and were collated using the program of Hewat¹⁵. Further data reduction and profile analysis were performed using the POWDER system¹⁶ on the Science Research Council's Interactive Computing Facility. The neutron scattering lengths used in the refinements were: $b(\text{Al}) = 0.35$, $b(\text{Si}) = 0.42$, $b(\text{O}) = 0.58$, and $b(\text{Tl}) = 0.89$ (all $\times 10^{-14}$ m)¹⁷.

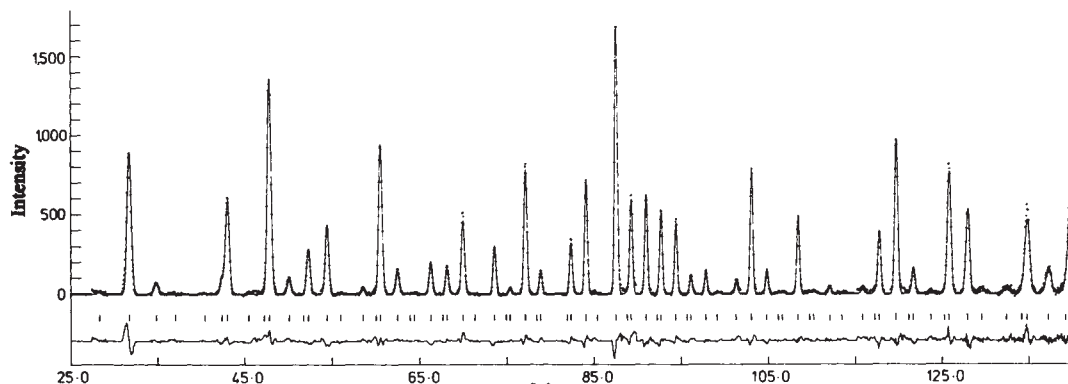
Results and discussion

The most striking aspect of the neutron powder pattern is the absence of any visual evidence for the rhombohedral distortion that was present in the anhydrous Na zeolite-A sample from which the Tl derivative was prepared. The conclusion that the sample is cubic was confirmed by the subsequent refinement that was carried out in space group Fm3c.

The Tl zeolite-A data were refined to a final R_{pr} of 11.33;

$$R_{\text{pr}} = \frac{\sum |y_i(\text{obs}) - \frac{1}{c} y_i(\text{calc})|}{\sum y_i(\text{obs})}$$

where $y_i(\text{obs})$ and $y_i(\text{calc})$ are the observed and calculated intensities, respectively, at the i th point on the profile, and c

Fig. 2 The observed (dots), calculated (solid curve) and difference profiles for Tl zeolite-A in Fm3c; reflection positions are also indicated.

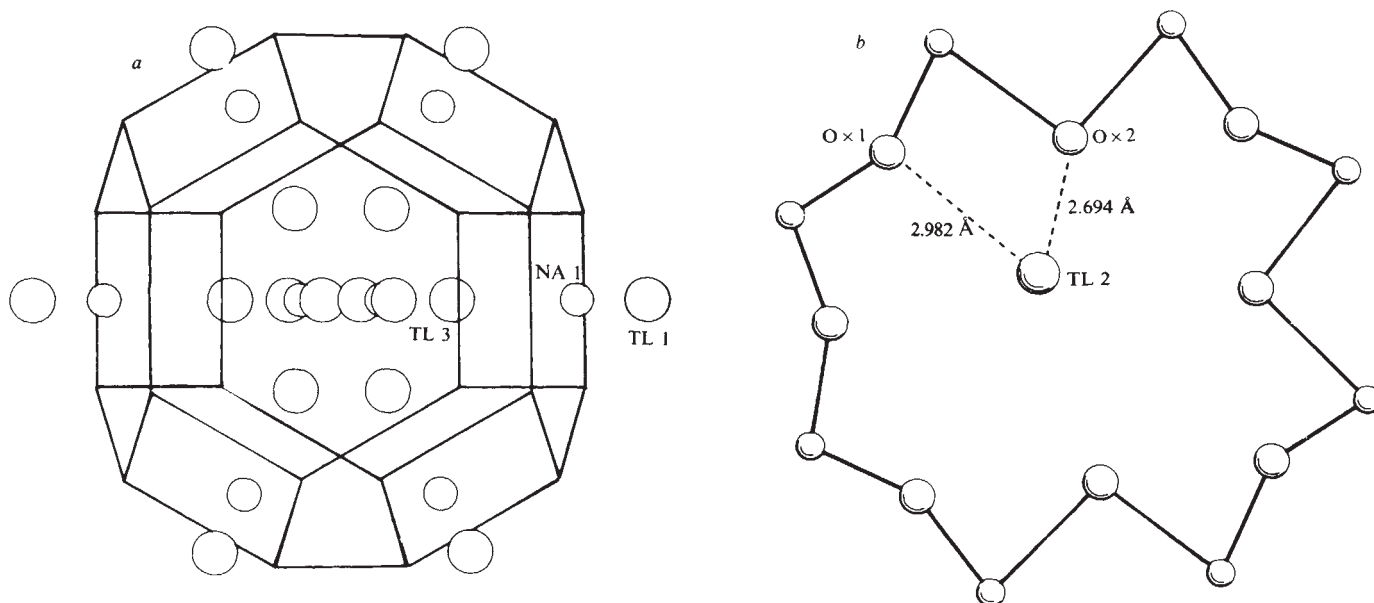


Fig. 3 a, The location of Tl(1), Tl(3) and Na(1) sites close to the 6-rings. b, The location of Tl(2) in the 8-ring.

is the scale factor. The final atomic coordinates and occupancy factors are given in Table 1, and the observed and calculated profiles are shown in Fig. 2. The lattice parameter obtained was 24.373 Å. The bond lengths given in Table 2 show that the Si–O and Al–O distances are unambiguously bimodal.

The ^{29}Si NMR spectrum of the Tl zeolite-A sample was essentially unchanged from that of the parent Na sample, confirming that the Si,Al distribution has been unaffected by the ion exchange reaction. The NMR result would seem to confirm the 3:1 ordering scheme for the aluminosilicate framework, and if this is indeed the case, then the neutron data should refine well in the cubic Pn3n model advanced by Bursill *et al.*, because this appears to be the only cubic model that is consistent with 3:1 ordering. The data were refined in this model to a final R_p value of 13.99%. The R_p value is thus >2% larger than the value obtained in Fm3c, even though four times as many atomic coordinates were refined in the Pn3n model.

The present neutron results point strongly towards the correctness of a 4:0 Si,Al ordering scheme. The bimodal Si–O and Al–O bond lengths are hard to rationalize with any other framework distribution, and the relatively poor refinement obtained in Pn3n would appear to be conclusive. If the Fm3c model is indeed correct for the Tl zeolite-A, then the position of the ^{29}Si NMR resonance at the chemical shift expected for 3:1 Si, Al ordering must be an anomaly that arises in zeolite-A because of the location of the Si in the double 4-rings. This conclusion has recently been corroborated by ^{29}Si NMR measurements on the zeolite ZK-4, which has the same aluminosilicate framework as zeolite-A^{18,19}. A further important feature of the present results is that the rhombohedral distortion is clearly dependent on the identity of the exchange-

able cations. If the 4:0 ordering scheme is correct, then the rhombohedral Na zeolite-A data should be refined in the appropriate space group for such ordering, R3c. The factors influencing the rhombohedral distortion in zeolite-A, and the origin of the violations of the *c*-glide condition in the cubic form, remain to be elucidated.

The Tl zeolite-A structure contains two cations, Tl(1) and Tl(3), on the 3-fold axis above and below the centre of the 6-ring (Fig. 3a). These cations are trigonally coordinated by oxygens in the 6-ring at 2.715 Å and 2.923 Å, respectively; the sum of the ionic radii for Tl^+ and O^{2-} is ~2.85 Å (ref. 20). Tl(2) is coordinated by two oxygens in the 8-ring, O(1) and O(2), at 2.982 Å and 2.694 Å, respectively (Fig. 3b). Cation (4) is found surprisingly close to the centre of the 6-ring at a distance of 2.256 Å from O(3). It essentially lies between Tl(1) and Tl(3) (Fig. 3a), at the position corresponding to the cation site with the highest occupancy in sodium zeolite-A. This evidence, together with the short cation(4)–oxygen distances, the small value of the lattice parameter, and the electron microscopy results, clearly confirms that cation(4) is in fact sodium arising from incomplete cation exchange. When the scattering length of sodium is substituted for that of Tl at this site, the total cation occupancy for the compound increases from 0.72(3) to 0.91(3); the theoretical value is 0.95(2). In earlier studies²¹ of ion-exchange at 25 °C, 64% conversion from the sodium to the thallium form was obtained; our results correspond to 66% conversion. The zero-coordinated Tl atom reported by Firor and Seff²² is not found in the present work.

We thank the SERC for neutron facilities at ILL and a studentship for M.M.E., Mr S. Heathman for experimental assistance, and the Exxon Research and Engineering Co. for a grant.

Received 28 May; accepted 8 July 1982.

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Specific protein–nucleic acid recognition in ribonuclease T₁–2'-guanylic acid complex: an X-ray study

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RNase T₁ is folded into an α -helix of 4.5 turns, covered by a four-strand antiparallel β -sheet. Specific recognition of 2'-guanylic acid arises from hydrogen bonding between main chain peptide groups and the O-6 and N-1-H of guanine, as well as from stacking of Tyr 45 on guanine. At the active site, Glu 58, His 92 and Arg 77 are involved in phosphodiester hydrolysis.

SPECIFIC recognition of certain nucleotides or nucleotide sequences in both RNA and DNA by proteins is of central importance in various processes in molecular biology. Understanding of the mechanism by which proteins express base specificity requires structural studies of model systems like nucleases and their complexes with nucleic acid fragments. So far, crystal structures of such systems have been published for bovine pancreatic ribonuclease A¹⁻³ and ribonuclease S⁴ and for the nuclease from *Staphylococcus aureus*⁵. While the staphylococcal enzyme hydrolyses both DNA and RNA, RNase S acts only on phosphodiester bonds at the 3'-terminal side of pyrimidine nucleotides in single-stranded RNA.

An even more selective enzyme, ribonuclease T₁ (RNase T₁; EC 3.1.27.3) from the fungus *Aspergillus oryzae*, degrades single-stranded RNA to yield exclusively oligonucleotides with terminal guanosine-3'-phosphate. As with RNase A, RNase T₁-catalysed cleavage involves formation of an intermediate, 2',3'-cyclic phosphate, followed by hydrolysis. Its marked specificity towards guanosine has not only made RNase T₁ an important tool in nucleic acid chemistry but it is also used extensively as a model compound to study the recognition of nucleic acids by proteins⁶.

Chemical modifications of RNase T₁, a small acidic protein, have demonstrated the importance of an active glutamic acid residue⁷ and of two histidines^{8,9} for its enzymatic activity. Participation of histidine residues in substrate binding and their interaction with carboxylate groups of the protein were also demonstrated by NMR spectroscopy^{10,11} and kinetic studies¹². However, the exact role of these residues (Glu 58, His 40 and His 92) in catalysis and/or base recognition is unknown. There is also some evidence for the side chain of the single arginine (Arg 77) being located near the active centre¹³ (see Fig. 1). The positions N-1, N-7 and the 6-oxo group of the guanine base appear to be recognized by RNase T₁.

The crystallographic investigation of the RNase T₁–2'-guanylic acid complex described here was undertaken to provide a structural basis for understanding the enzyme's remarkable base specificity, and the molecular mechanism of RNase T₁-catalysed RNA cleavage.

Structure determination

As described previously¹⁴, RNase T₁ co-crystallized with 0.25% 2'-guanylic acid from sodium acetate buffer adjusted to pH 4.0–4.4 when dialysed against 53% 2-methyl-2,4-pentanediol. Crystals used in X-ray diffraction work were orthorhombic, P2₁2₁2₁, with cell parameters $a = 46.8$ Å, $b = 50.2$ Å and $c = 40.4$ Å. Heavy atom derivatives were prepared by soaking

protein crystals in 1–5 mM solutions of the respective reagent in the mother liquor.

Diffraction data were collected by a diffractometer in the ω -scan mode with stationary background counts on both sides of each reflection. Five individual data sets of native RNase T₁ and two sets of both Pb(II) acetate and Pt(NH₃)₄Cl₂ derivatives including Friedel pairs, were measured using one single crystal for each set. Reflection standard deviations were computed from counting statistics¹⁵ and the data sets were placed on a common scale after semi-empirical absorption correction¹⁶. Corresponding structure factor amplitudes were then averaged, weighted by the inverse square of their standard deviations.

Estimates of the heavy atom structure factor amplitudes¹⁷ were used to locate the heavy atom sites by Patterson syntheses and direct methods¹⁸. Heavy atom parameters were subjected to full matrix least-squares refinement and finally the enantiomorph was selected by calculating difference Fourier maps based on protein phases derived from either hand of the sites

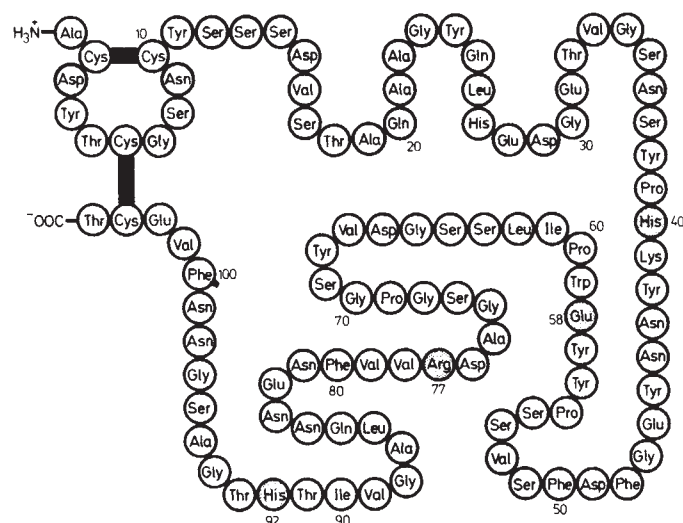


Fig. 1 Covalent structure of RNase T₁ (ref. 21). Residues shown shaded are critical for enzymatic activity. The protein used in this investigation was an isoenzyme of the sequenced species, having one additional lysine residue and lacking one glutamine or glutamate⁴⁸. Preliminary results of the amino acid analysis of iso-RNase T₁ (H. Kratzin, personal communication) suggest that Gln 25 is exchanged with Lys. The present X-ray study showed Gln 25 or Gln 85 as possible exchange positions. Enzymatic activity of the iso-RNase T₁ is the same as that of the mother protein.

Table 1 Data collection and processing statistics

Derivative	Minimum resolution (Å)	Reflections measured*	Unique reflections*	R_M	R_1	R_A	Heavy atom sites	$R_{F_{HLE}}$	Phasing power
Native RNase T ₁	2.5	14,318	3,550	0.098	—	—	—	—	—
Pb(II) acetate	2.5	5,793	3,547	0.066	0.272	0.109	6	0.394	2.327
Pt(NH ₃) ₄ Cl ₂	2.5	4,897	3,139	0.051	0.159	0.125	2	0.372	2.056
Pb/Pt	3.0	1,970	1,970	—	0.149	0.108	3	0.377	2.259

Preparation of heavy atom derivatives: Pb(II) acetate, crystals were soaked for 1 day in a 1 mM solution; Pt(NH₃)₄Cl₂, 2 days in a 5 mM solution; double derivative (Pb/Pt), first 1 day in 1 mM Pb(II) acetate, then 2 days in 5 mM Pt(NH₃)₄Cl₂. Individual R values are defined as:

$$R_M = \frac{\sum_i |F_i - F_i^*|}{\sum_i F_i}, \quad R_1 = \frac{\sum |F_P - F_{PH}|}{\sum F_P}, \quad F_A = \frac{\sum |F^+ - F^-|}{1/2 \sum |F^+ + F^-|}, \quad R_{F_{HLE}} = \frac{\sum |F_{HLE} - F_{H(calc)}|}{\sum F_{HLE}}$$

Summations are over all reflections. F_P is the protein structure factor amplitude, F_{PH} the derivative structure factor amplitude, F^+ and F^- the positive and negative equivalents for Friedel pairs. F_{HLE} and $F_{H(calc)}$ are the estimated¹⁷ and calculated heavy atom structure factor amplitudes, respectively. The phasing power is defined as $\sum F_{H(calc)} / \sum E_{best}$, where E_{best} is the lack of closure error at the centroid protein phase.

* For derivatives, Friedel pairs were counted as one reflection.

of the platinum derivative. Conventional Blow and Crick phase determination¹⁹ yielded a mean figure of merit $\langle m \rangle = 0.75$ for all 3,547 reflections to 2.5 Å. Data processing and heavy atom refinement statistics are given in Table 1.

Map interpretation

An electron density map was calculated for protein phases obtained as described above and sampled at grid spacings of 0.9 Å. Positive density was contoured at a scale of 1 Å = 2 cm on transparent plastic sheets, which were placed in an optical comparator²⁰ for the construction of a Kendrew wire model of the RNase T₁-2'-guanylic acid complex.

From a first inspection of the map, the molecular boundaries of the ribonuclease molecule and the bound nucleotide were immediately apparent. The base, ribose and phosphate portions of 2'-GMP were well resolved. Once the chain termini were identified, tracing of the protein polypeptide chain was straightforward. In general, carbonyl 'bumps' were visible and in the pleated sheet structures, some hydrogen bonds were indicated by weak electron densities. Most amino acid side chains are clearly defined and in agreement with the published sequence²¹. The side chain of Lys 41 which points towards the solvent, however, has no density beyond its C^β atom, probably a consequence of disorder. Coordinates of the 801 atoms of the complex were directly determined from the model and will be deposited with the Brookhaven Protein Data Bank.

RNase T₁ is a compact molecule

As illustrated in the schematic drawing in Fig. 2, the RNase T₁ molecule is a compact, globular protein whose shape may be defined as a slightly distorted tetrahedron with corner positions

occupied by the side chains of residues Ala 1, Glu 31, Asn 83 and Ser 96. Molecular dimensions are 32 × 33 × 30 Å measured along the unit cell axes. Individual RNase T₁ molecules in the crystal lattice are largely separated by solvent regions, with only three obvious intermolecular contacts involving one hydrogen bond between the side chains of Glu 46 and Ser 35.

The polypeptide chain connecting amino acid residues adjacent to the amino-terminus almost forms a closed loop, which is held together by the disulphide bridge between residues 2 and 10. The chain then enters a regular α-helix of ~4.5 turns, followed by two wide loops separated by a stretch of extended structure to which the inhibitor is bound. Three strands of antiparallel pleated β-sheet follow, with another loop inserted after the first strand. The structure is completed by a wide turn, bringing the carboxy-terminus into the vicinity of the amino end and closing the disulphide bridge 6-103.

While the α-helix is entirely regular, the pleated β-sheet structure is severely bowed and twisted in a left-handed sense. The hydrogen-bonding pattern between backbone segments Asp 76 to Asn 81 and Asn 84 to Ile 90 is disturbed by insertion of residues in the latter strand, residue Val 79 forming a classical 'β-bulge'²² with the dipeptide Ala 87...Gly 88. Another peculiarity of RNase T₁ concerns proline residues 39 and 55 which fit the electron density best when in *cis* form.

The globular structure of the RNase T₁ molecule hides a hydrophobic core located mainly in the interface region between the long α-helix and the pleated sheet. Therefore, all hydrophobic side chains of RNase T₁ are at least partially protected against solvent contact.

Unlike bovine pancreatic ribonuclease, RNase T₁ does not provide a distinct cleft for substrate binding. In view of the outstanding specificity of RNase T₁, it is surprising that the inhibitor base is bound merely on the enzyme surface, while the 2'-GMP phosphate portion lies in a shallow concave depression. A characteristic assembly of aromatic side chains around the active site region might be involved in sub-site interactions with adjacent nucleotides along the polynucleotide chain; this would be in agreement with kinetic studies^{23,24} which have revealed differences in the hydrolysis of GpN substrates.

Positive charges cluster in a narrow zone

It is an apparent paradox that RNase T₁ is an acidic protein exhibiting an isoelectric point of 3.8 (ref. 25), while its function is to bind to and degrade negatively charged nucleic acids. It shares this property with ribonuclease U₂ (from *Ustilago sphaerogena*⁶) and ribonuclease St from *Streptomyces erythreus*²⁶, but not with the ribonucleases from *Bacillus amyloliquefaciens* and *Bacillus intermedius*, which belong to the same sequence-related family of enzymes²⁷. On complex formation, some of the counter-cations of the RNA must be replaced by the ribonuclease to allow close intermolecular contacts, followed by cleavage of the phosphodiester bond. Therefore it is of interest to examine the distribution of charges on the molecular surface of RNase T₁.

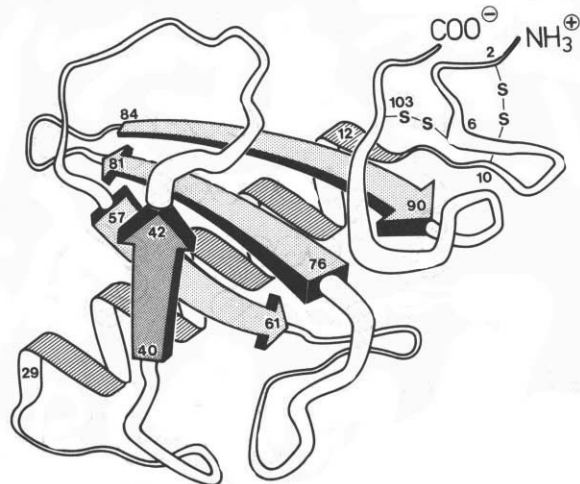


Fig. 2 Schematic drawing of the RNase T₁ polypeptide folding. Taking into account residues which occur in reverse β-turns, half the molecule consists of well-defined secondary structure. Numbers refer to amino acids in the primary sequence (see Fig. 1).

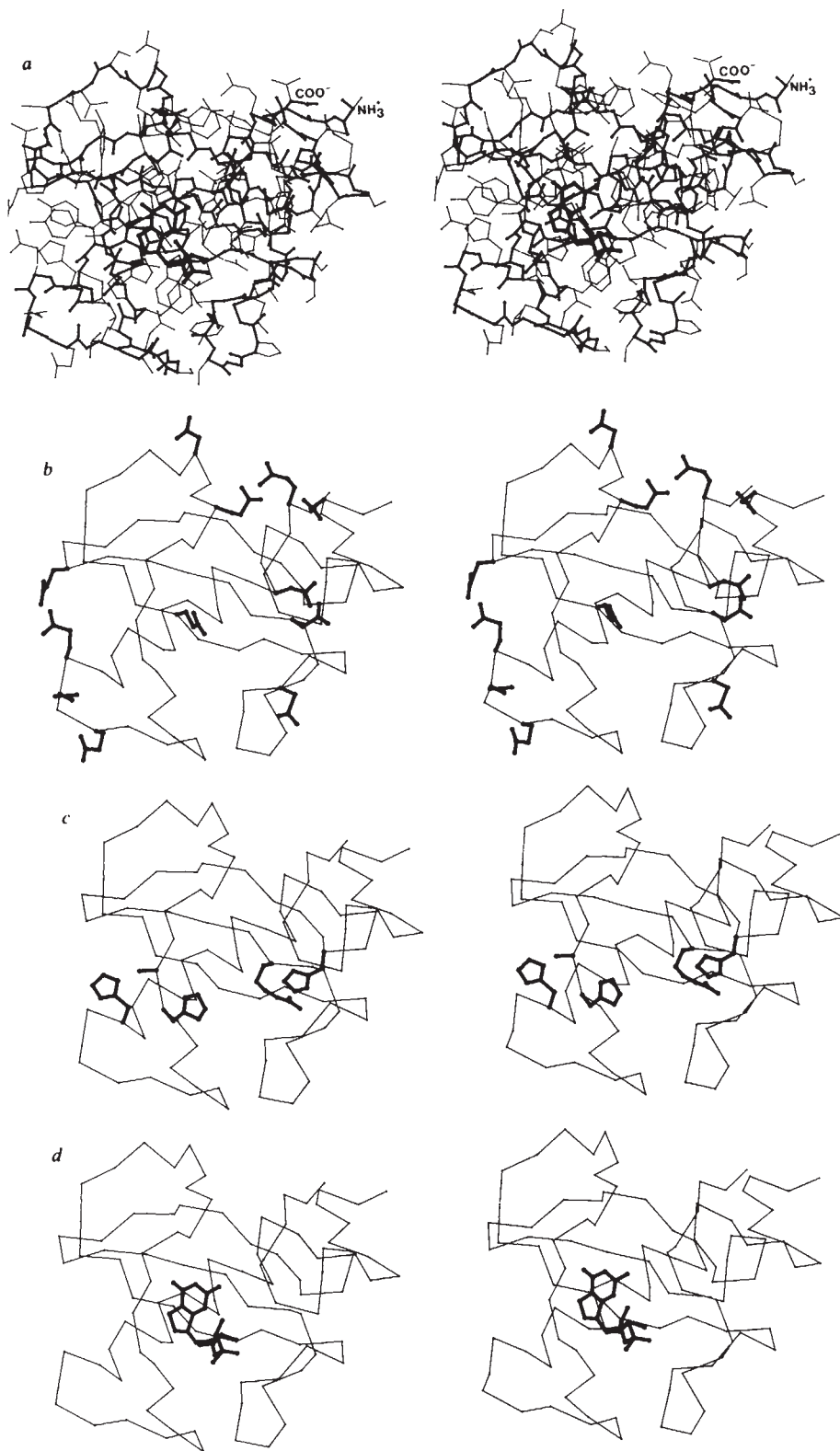


Fig. 3 Stereo drawings of the RNase T₁ molecule. *a*, The whole RNase T₁-2'-GMP complex with the protein backbone and the nucleotide indicated by heavier lines. *b*, C^α-backbone and acidic residues. *c*, C^α-backbone and basic residues. The side chain of Lys 41 is not visible in the electron density map and is indicated by its C^β-atom only. *d*, C^α-backbone with 2'-guanylic acid shown by heavier lines.

At pH values of ~5, where RNase T₁ has maximum affinity for small substrate analogues²⁸, most carboxylate groups are deprotonated, while the basic residues including the histidines bear a positive charge. The resulting charge distribution of the RNase T₁ molecule is shown in Fig. 3*b,c*. Except for the side chain of Glu 58, all charged residues are located on the molecular periphery, the acidic groups being equally distributed over the whole surface. The side chains of the basic residues His 40, Arg 77 and His 92, however, are arranged in a narrow zone around the active site (see below) and form the environment for the binding of the inhibitor phosphate group. On this

basis, it is tempting to deduce a function for the charged residues of RNase T₁ in promoting the attachment of its shallow active site groove to the negatively charged nucleic acid via electrostatic interactions.

Protein-nucleic acid recognition is specific

In the complex formed between RNase T₁ and 2'-GMP, the ribose moiety of the nucleotide adopts a C-3'-*endo* type pucker, the orientation about the C-4'—C-5' bond is *+gauche* and the torsion angle defined by the glycosyl link is in the *syn* range.

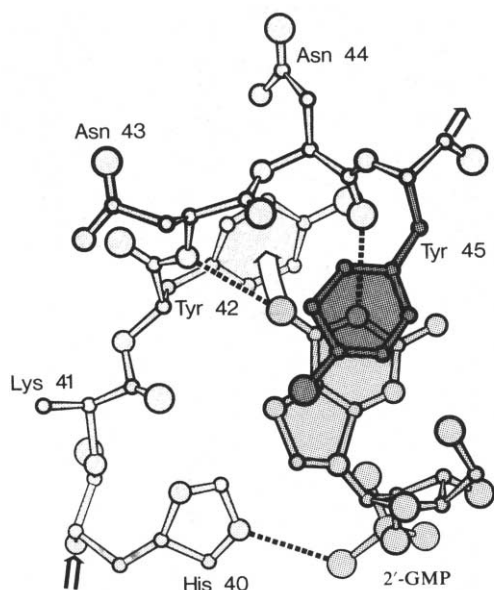


Fig. 4 Base recognition by RNase T₁. 2'-GMP is shown together with the portion of the RNase T₁ polypeptide chain from His 40 to Tyr 45. Note the hydrogen bonds (broken lines) between base positions N-1 and O-6 and the protein backbone and between the His 40 imidazole and the inhibitor phosphate moiety. The wide curved arrow indicates interactions between the O-6 of guanine and the side chain of Tyr 42. The phenyl ring of Tyr 45 stacks parallel to the guanine plane, 3.5 Å distant. The electron density map shows some evidence of an alternative, non-stacking position of the side chain of Tyr 45 which would require rotation around the C^α-C^β and C^β-C^γ bonds.

The latter conformation is unusual but not unexpected. Thus, it has been proposed earlier on the basis of circular dichroism spectra²⁹ that guanosine nucleotides bind to RNase T₁ in this particular form and there is evidence for easy conversion from *anti* to *syn* conformation of guanosine, the latter even being preferred in acidic medium^{30,31}.

The guanine base of the inhibitor is bound to the polypeptide backbone of RNase T₁ by hydrogen bonds between the N-1—H of guanine and the main-chain carbonyl of Asn 44 and between O-6 of guanine and the main-chain —NH of Asn 43 as shown in Fig. 4. This result is consistent with the requirement of the guanine N-1 and O-6 positions for base recognition by RNase T₁ (ref. 6). The geometry of the guanine-RNase T₁ interactions also suggests that substitution of the guanine base at N-7 would interfere sterically with the backbone carbonyl group of Lys 41, and that protonation of N-7 by the enzyme, as suggested on the basis of proton NMR studies¹¹, is unlikely. This is because, first, we should observe strong hydrogen bonding between guanine N-7 and RNase T₁ (this is not found) and second, the *pK_a* of guanine N-7, 2.8, is too low to induce protonation in crystallization conditions at pH 4.0–4.4. A NMR investigation of the binding of ¹⁵N-enriched 3'-GMP to RNase T₁ (ref. 32) also did not support the proposed N-7 protonation; this experiment has been interpreted in terms of a strong interaction of the amino group at guanine C-2 with RNase T₁. In the X-ray structure, however, no close contact with the guanine amino group is observed, therefore it is not essential for RNase T₁-guanine recognition. This finding is in agreement with chemical studies demonstrating that substrates in which inosine is substituted for guanosine are converted at almost the same rate⁶.

In addition to these hydrogen-bonding contacts, our model indicates interactions between guanine O-6 and the phenyl moiety of Tyr 42 and, more importantly, stacking between the guanine base and the phenyl residue of Tyr 45. The latter lies 3.5 Å 'above' the base at the very periphery of the complex. As the map shows a neighbouring portion of weak electron

density probably representing an alternative location of the Tyr 45 side chain, a conformational change of RNase T₁ on substrate binding is probable, swinging Tyr 45 over and into a stacking position with the guanine base. This movement locks in guanine after it has entered the active site following recognition, by hydrogen bonding to the main-chain atoms of Asn 43 and Asn 44. In agreement with these observations, earlier studies showed that the fluorescence of one or two tyrosines is affected when RNase T₁ is complexed with substrate analogues³³, and the above-mentioned NMR results should be reinterpreted in this light.

There is no apparent interaction between RNase T₁ and the ribose of 2'-GMP but the phosphate is involved in hydrogen bonding to the imidazole of His 40. As the phosphate group is near the side chains of His 40, Glu 58, Arg 77 and His 92 which line the active site of RNase T₁ (see below), it is possible that the more natural analogue 3'-GMP would involve other interactions of its ribose and phosphate portions. Thus, slight rotation about the glycosyl link would bring the ribose O-5' hydroxyl in hydrogen-bonding contact with Asn 98, and the 3'-phosphate would be in a position to hydrogen bond with His 92 and also with Arg 77 if this side chain were rotated into another position.

In this regard, it is of interest that the *anti*-fixed nucleotide derivative 5'-deoxy-8,5'-cyclo-guanosine-2',3'-cyclophosphate is bound to RNase T₁ and is converted to the corresponding 3'-phosphate³². As the rate of hydrolysis is rather slow compared with guanosine-2',3'-cyclophosphate, we assume that the base binds nonspecifically to the enzyme (probably promoted by stacking with Tyr 45) whereas the sugar-phosphate moiety protrudes into the active site and is cleaved. This interpretation also explains why dinucleoside phosphates with other than guanosine in the 5'-position are hydrolysed by the enzyme, although at dramatically reduced rates (a factor of $\leq 10^{-6}$) (ref. 6).

Why is the protein main chain involved in specific recognition?

When considering the specific protein-nucleic acid recognition, the often discussed side chain-base interactions spring to mind^{34,35}. In the case of guanine, these could involve Asp or Glu carboxylate accepting hydrogen bonds from guanine N-1—H and N-2—H, Arg guanidinium donating hydrogen bonds to N-7 and O-6, or Gln, Asn amide groups donating and accepting hydrogen bonds to O-6 and from N-1—H, respectively. It is surprising to find that in the RNase T₁-2'-GMP

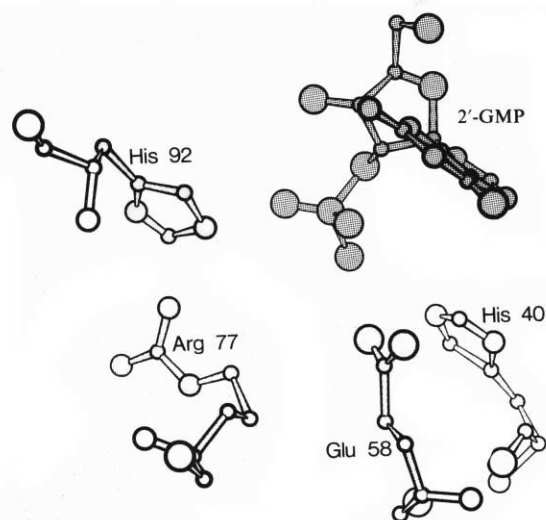


Fig. 5 Catalytic centre of RNase T₁. The plane of the guanine base is almost perpendicular to the plot.

complex none of these interactions is observed and that the nonspecific main chain peptide groups have been selected for specific recognition.

Why is this so? One reason could be that amino acid side chains are floppy because of rotations about C—C single bonds, and therefore do not provide for a well-determined matrix necessary for fast, highly specific mutual recognition of the two partners. Alternatively, the main chain can be adjusted and maintained in any conceivable configuration, with peptide groups in fixed positions and ideally suited as hydrogen bonding donors and acceptors. This allows for much better fine-tuning of these interactions than utilization of amino acid side chains. Furthermore the folding of the chain can be designed to be stiff or flexible, depending on the respective needs to be served.

Catalytic site geometry suggests mechanism of RNA hydrolysis

The catalytic centre of RNase T₁ is shown in Fig. 5. His 40, in the immediate neighbourhood of the side chain of Glu 58, forms a hydrogen bond or salt bridge with the 2'-GMP phosphate group. The His 92 imidazole ring points towards the phosphate portion of the inhibitor, but is too far away to interact directly with it, whereas the adjacent guanidinium group of Arg 77 is turned away from the active site. The side chain of Asn 98 is close to the 3'-hydroxyl group of the nucleotide.

Proton NMR spectroscopy has revealed unusually high *pK_a* values (7.2–7.9) for two or three histidines of RNase T₁ (refs 36–38). The interpretation that these residues interact with acidic side chains of the protein is supported by the crystal structure, which shows His 27 located close to both Glu 28 and Glu 82 and His 40 near the Glu 58 carboxyl group. Interaction of His 92 with an acidic group¹², however, would require an almost complete re-folding of the protein. The interaction of His 40 with Glu 58, as observed in the electron density map, has also been deduced from NMR studies²⁸. As the side chain of Glu 58 is close to the inhibitor phosphate moiety it is presumably protonated. This is consistent with the result of potentiometric titrations of RNase T₁, which demonstrated that the *pK* of one carboxylate group is shifted from 4.9 to 7.8 on binding of 2'-guanylic acid³⁹.

Assuming a similar binding of true substrates to RNase T₁, we propose a catalytic mechanism for the enzyme where, in the transphosphorylation step, the carboxylate group of Glu 58 acts as a base in abstracting the proton from the ribose O-2' and the imidazole of His 92 protonates the leaving O-5'. The function of His 40 is merely to activate the Glu 58 carboxylate group. Obviously, this histidine is not conserved in all members of the RNase T₁ family²⁷, because it is not essential for catalysis. This mechanism for the transphosphorylation step is consistent

with an in-line mechanism⁴⁰ for RNase T₁ action as proposed by Eckstein *et al.*⁴¹ on the basis of the methanolysis of cyclic phosphorothioates.

The situation outlined above resembles that found in bovine pancreatic RNase A, where histidine residues 12 and 119 serve as general acid and base catalysts⁴². The function of the Arg 77 of RNase T₁ could be similar to that of Lys 41 of RNase S—that is, to stabilize the pentacovalent intermediate of transphosphorylation through electrostatic interactions⁴³.

Relationship of RNase T₁ to other nucleases

The folding of RNase T₁ bears no similarity to that of *Staphylococcus* nuclease or of RNase A from bovine pancreas. In the complex between the nonspecific staphylococcal nuclease and thymidine 3',5'-bisphosphate⁴⁴, no specific interaction between the protein and the base is observed. On the other hand, X-ray diffraction studies of several inhibitors bound to RNase S^{42,43} have revealed that the pyrimidine base forms hydrogen bonds between its N-3 atom and O^γ1 of a threonine residue and between its O-2 and the backbone nitrogen of the same residue. The latter bond does not occur in all inhibitor complexes investigated⁴³. The threonine hydroxyl group serves as a hydrogen bond acceptor with uracil, where N-3 is protonated, and as a donor in the case of cytosine, thus accommodating both pyrimidine nucleotides. If mechanisms of hydrolysis of the three enzymes are compared, we find that those of RNase A and of RNase T₁ are similar to one another but different from that of staphylococcal nuclease.

Recently, the chain foldings of the ribonucleases from *S. erythraeus*²⁶ and *B. amyloliquefaciens*⁴⁵ have been elucidated by X-ray crystallography. They largely resemble that found for RNase T₁, with a long helix covered by a four-strand antiparallel pleated β -sheet. These enzymes also display sequence homologies with RNase T₁, especially around the active site residues Glu 58, Arg 77 and His 92 (ref. 27) of RNase T₁, which are found in a comparable arrangement in all three proteins. The importance of the active glutamic acid for the action of the *Streptomyces* enzyme has been demonstrated⁴⁶. We conclude, therefore, that the above enzymes share a common mechanism of nucleic acid hydrolysis.

To obtain a more precise description of the processes involved in RNase T₁ action, we have commenced refinement of the present model and data collection for higher resolution. The structures of the free enzyme and of complexes with guanosine 3',5'-bisphosphate and 2'-deoxy-2'-fluoroguanlyl-(3'-5')-uridine⁴⁷ will be investigated in parallel.

All computations were carried out on the UNIVAC 1100/82 of the Gesellschaft für Wissenschaftliche Datenverarbeitung, Göttingen. We thank Professor Rüterjans and co-workers for supplying purified RNase T₁.

Received 25 May; accepted 2 July 1982.

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Sequence specificity of trinucleoside diphosphate binding to polymerized tobacco mosaic virus protein

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The binding of trinucleoside diphosphates to long helical rods of tobacco mosaic virus (TMV) protein is shown to depend on base sequence, 5' AAG 3' binding being the strongest of the 25 trinucleoside diphosphate sequences measured. As TMV has a stoichiometry of three nucleotides per protein subunit, the sequence of TMV RNA suggested to be the nucleation site for self-assembly of the virus has three possible binding frames. From our binding constant data the most likely frame is predicted and shown to have two contiguous AAG sequences in a hairpin loop region.

THE mechanism of spontaneous self-assembly of tobacco mosaic virus (TMV) from its component RNA and single coat protein (TMVP) has been the subject of increasingly detailed studies. New information about the role of various TMVP aggregation states in determining the dynamics of the self-assembly process and about the molecular structure of TMVP, the whole virus and the RNA have made it possible to enquire into the chemical and structural basis for the initial recognition events between the protein and RNA.

It has been shown that TMV self-assembly proceeds by three overall processes. There is a nucleation process which involves initial binding of the protein on the RNA at a site that is ~1,000 nucleotides from the 3' end¹. Subsequent growth of the nucleoprotein complex is bidirectional and occurs by an elongation reaction involving the addition of protein to the RNA as the helical rod grows in the 5' direction, and a second elongation reaction resulting from protein addition in the 3' direction^{2,3}.

Reconstitution experiments using intact RNA and limiting amounts of TMVP, predominantly in the form of 20S two-turn (disk $\rightleftharpoons$ helix) protein, have demonstrated the formation of short, partially assembled rods containing RNA that is nuclease-resistant and derived from the internal assembly nucleation site⁴. The nucleotide sequence of the protected portion of the RNA has been determined⁵ and overlaps much of the sequence determined in an independent effort which used a different protection strategy, that is, binding of aggregates of TMVP to

fragments of TMV RNA generated by limited ribonuclease digestion⁶. These two studies have led to two different proposals of a stable hairpin-loop secondary structure for the RNA at the nucleation site. The hairpin-loop structure is proposed to be important for the nucleation of TMV assembly because it could allow the RNA to bind from within the hole of the initiating two-turn (disk $\rightleftharpoons$ helix) protein structure⁷. In addition, studies of the nucleotide sequences, under the constraint that the stoichiometry of the TMV particle (three nucleotides of the RNA molecule per 17,530-molecular weight (MW) protein subunit) be preserved, have led to the proposal that trinucleotide sequences at the assembly nucleation site form a reading frame for the initial binding of the 20S aggregate of TMVP^{5,6}.

We report here the results of equilibrium dialysis binding experiments utilizing various ³H-labelled trinucleoside diphosphates (trimers) and helical rods of TMVP formed in weak acid conditions. These experiments were undertaken to determine whether there are trinucleotide sequences that interact preferentially with TMV protein, and, if so, to quantitate the free energy contribution of trimer binding to helical rods of TMV protein to the total free energy of nucleation of TMV self-assembly. The binding measurements reveal a strong sequence specificity with respect to the strength of trimer binding, with AAG being the strongest ($12 \times 10^3 \text{ M}^{-1}$). These results suggest that the initial protein-nucleic acid recognition probably occurs in a hairpin-loop region of the RNA and has the binding frame —CAG/AAG/AAG—. This frame of the sequence can provide ~16 kcal of binding energy per mole of sites for the nucleation reaction in TMV self-assembly.

Dependence of binding on trimer sequence

Our initial attempts to measure trinucleotide binding to TMV protein, carried out in collaboration with Professor O. C. Uhlenbeck (University of Illinois), were performed at pH 7.0. At this pH the protein exists at 0 °C as a 4S species (approximately a trimer of the 17,530-MW protein subunit) or at 20 °C as an equilibrium mixture of 4S and 20S sedimenting boundaries. The latter boundary represents a mixture consisting of two-turn disks of 34 monomers⁸ and short helices, probably about two turns long⁹. In these conditions no trimer binding was detected at the lower temperature. At 20–25 °C, however, very weak binding was detected by Professor Uhlenbeck for AAG, CAG and UAG (all nucleotide sequences given here are written 5'→3'). Subsequent initial binding experiments at pH 6.5, 20 °C, in which the protein existed as short helical rods¹⁰, revealed that the trinucleoside diphosphates AAG, CAG, UAG and GAA bind weakly with apparent binding constants in the range 200–300 M⁻¹ (J.J.S. and T.M.S., unpublished results).

The pH dependence of the binding between pH 6.0 and 7.0 may result from protonation of the carboxyl groups shown in

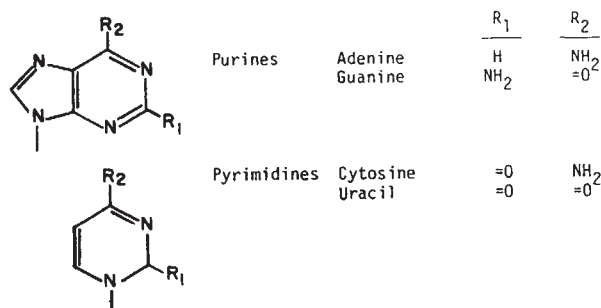


Fig. 1 Influence of single base substitutions in the trimer XAG on the apparent free energy of binding, ΔG^* .

	$\Delta(\Delta G^*)$ (kcal mol ⁻¹)
GAG-UAG	-0.46 ± 0.1
AAG-CAG	-0.42 ± 0.1
AAG-GAG	-0.64 ± 0.1
CAG-UAG	-0.68 ± 0.1

For the base in the 5' position the binding energy is more sensitive to the NH₂ and the =O ring substitutions than it is to the size of the heterocyclic ring.

Table 1 Apparent binding constants, $10^{-3} \times K$ (M^{-1}), of various trinucleoside diphosphates to helical rods of polymerized TMVP

Trimer (5' → 3')	Method of dialysis	
	I	II
AAA	0	0
AAG	11.7 (0.2)	12.0 (0.2)
GAA	4.4 (0.4) (pH 5.48)	4.7 (0.1)
AGA	1.0 (0.1)	1.0 (0.2)
GAG	—	4.0 (0.3)
UAG	1.7 (0.1)	1.9 (0.1)
UGA	0.8 (0.2) (pH 5–5.5)	—
GAU	0	—
AGU	0	—
AUG	—	4.1 (0.3)
CAG	5.9 (0.2)	5.8 (0.2)
CGA	0.75 (0.05) (pH 5.27)	—
GAC	0 (pH 5.31)	—
ACG	0.45 (0.05) (pH 5.46)	—
AAU	0	—
UAA	0 (pH 5.31)	—
UUU	0	—
CCU	0 (pH 5.33)	—
UUG	1.2 (0.1)	—
GUU	0 (pH 5.62)	0 (pH 5.56)
AUU	0	0
ACU	0	0
CCA	0	—
CUA	0	—
CGU	0 (pH 5.10)	—

Temperature, $20 \pm 0.1^\circ C$. Binding site concentration = $5.6 \pm 0.3 \times 10^{-4} M$ (the error denotes the range of concentrations over which different experiments were performed and not the error in any single measurement of the protein concentration). pH values are 5.40 ± 0.05 except where indicated. In most cases the binding constants are the average of those determined in one experiment from two or more samples taken at dialysis equilibrium. The errors reported (in parentheses) represent the range of binding constants determined in this way. The K values for AGA and GAG represent averages of several equilibrium measurements in two completely separate experiments; the errors indicate the range of values over the two experiments. Equilibrium dialysis experiments were performed in two ways; binding of trimers to TMV protein rods formed during (method I) or before (method II) dialysis. Method I: a solution of TMV protein as 4S protein in a buffer of KH_2PO_4 (0.033 M), K_2HPO_4 (0.017 M), and KCl (0.12 M), pH 6.47 ($24^\circ C$), was slowly warmed from $0^\circ C$ to $20^\circ C$ resulting in the formation of short helical rods as described by Shire *et al.*³¹. When examined in the analytical ultracentrifuge at $22^\circ C$, three boundaries were observed with sedimentation coefficients, $s_{20,w}$, and weight fractions, respectively, of 33–35S, 0.17–0.20; 23–24S, 0.72–0.74; and A-protein: 4S, 0.06–0.09. A 50- μl sample of the protein was equilibrated with ~ 0.3 – $0.5 \mu Ci$ of trimer (final total concentration at dialysis equilibrium between 0.1 and $1 \mu M$) for 12–18 h at room temperature (23° – $25^\circ C$), then loaded into a dialysis chamber. In the opposite chamber was placed 50 μl of a pH 4.97 ($23^\circ C$) buffer consisting of acetic acid (0.029 M), sodium acetate (0.071 M), KH_2PO_4 (0.050 M) and KCl (0.16 M). Method II: preformed TMV protein rods were formed as in method I but the trimer was not included with the protein. After 24 h of dialysis at $20^\circ C$, the dialysates were withdrawn from the buffer chambers and added individually to lyophilized trimers. The trimer-dialysate solutions were then loaded back individually into the empty dialysis chambers to allow the trimers to dialyse into the chambers containing the preformed protein rods. In most cases the first samples were withdrawn 4–5 days after the start of the experiment. In both methods I and II, pH values of solutions removed from the chambers at the end of an experiment were determined with a pH microelectrode. Details of the dialysis chambers used here and the method of equilibrium microdialysis are described elsewhere³². The total molar site concentration of TMV protein was taken to be the same as the total subunit concentration at the start of dialysis. It was determined spectrophotometrically using an extinction coefficient of $1.30 (mg ml^{-1})^{-1} cm^{-1}$ (ref. 33) and a molecular weight of 17,530 per subunit³⁴. 3H -labelled trimers of specific activity between 6 and 16 Ci mmol⁻¹ were synthesized according to the method of Thach and Doty³⁵. Precautions were taken to prevent ribonuclease degradation of the trimers³⁶ and all samples were checked for purity by paper chromatography³⁵ after synthesis, and for degradation after equilibrium dialysis. TMV protein prepared as described by Shire *et al.*³⁷ was found by Professor Uhlenbeck to be ribonuclease-free. Because the ratio of molar concentration of sites to the molar concentration of bound plus unbound trimer at dialysis equilibrium was ≥ 500 , the binding constants were calculated from the relation, $K = (R - 1)/C_0$, which assumes binding to one class of independent sites. C_0 , total molar site concentration; R , ratio of c.p.m. in 3 μl withdrawn from the chamber containing the protein to c.p.m. in 3 μl withdrawn from the opposite (dialysate) chamber. Binding constants < 200 – $300 M^{-1}$ are not measurable within experimental error by this method.

the X-ray diffraction structure of TMV to be near the nucleotide binding sites¹¹. To enhance trinucleoside diphosphate binding we used a lower pH (5–6) to reduce the net negative charge on the protein through binding of the polymerization-linked protons¹². The titration curve of TMV protein near $20^\circ C$ shows

that one proton is bound when the pH is reduced from 8.0 to 6.5 and another on reduction of the pH from 6.5 to 5.5 (refs 13–17). As trinucleoside diphosphates are negatively charged in this pH range, a significant pH-dependence of binding was expected for weak binding.

In addition to reducing the net negative charge on the protein, the binding of the two polymerization-linked protons switches the subunit conformation in the 20S disk aggregate¹⁸ to one that is very similar to that in the helical subunit array found in the whole virus^{19,20}. On binding these polymerization-linked protons, TMVP subunits polymerize to form virus-like long helical rods^{21,22}. The greater affinity of the trimers for polymerized TMV protein at pH < 6 relative to the weak binding of some of them to the protein aggregates at pH 6.5 and 7.0 (see Table 1) is considered to result from the presence of an ordered structure that exists at pH < 6 in the polypeptide chain at low radius between the centre of the rod and the region where the RNA binds to each subunit. This ordered structure has also been found by X-ray diffraction to exist in TMV¹¹ and in helical rods of polymerized TMVP formed at pH 5.5 (refs 19,20). However, the crystal structure of the 20S disk aggregate¹⁸ reveals disorder of the polypeptide chain at low disk radius.

As TMV protein subunits polymerize extensively into helical rods at $20^\circ C$ and pH < 6 (the conditions in which the present systematic binding measurements were made), we used intact virus to test for nonspecific binding. Neither of two trimers that bind to the protein, AAG or CAG, show binding to intact TMV in the same conditions. Binding of the trimers to the natural nucleotide sites on the native virus is precluded by the presence of the RNA, therefore the lack of binding of AAG and CAG to TMV means that there are no trivial trinucleotide binding sites on the coat protein of the virus. The similarity of the protein subunit packing in the virus and the helical protein rods^{19,20,23} leads us to assume that RNA-free TMV protein rods also do not have trivial trinucleotide binding sites.

Table 1 gives the apparent association equilibrium constants (K) for the binding of 25 trimers to TMV protein rods. In all cases the binding (or lack of it) was the same to preformed protein rods (method II) as to rods formed in the presence of trimers (method I), thus we can conclude that no nonspecific entrapment of trimers occurs during protein polymerization in method I. In general, the binding constants were found to be independent of the way in which the experiment was performed, indicating that the binding sites are structurally alike in both types of TMV protein rod preparation. At the concentrations of trimers used, less than 0.2% of the total trinucleotide sites on the protein rods were occupied and no site interactions were detectable.

The shape of the titration curve of TMV protein at $20^\circ C$ suggests that the net charge per subunit between pH 5 and 6 depends strongly on the pH (refs 13–17). As the trinucleoside diphosphates are negatively charged in this pH range we would expect, and indeed we have observed, that the measured binding constants increase with decreasing pH. The binding constant for AGA at pH 5.25 is $1.6 \times 10^3 M^{-1}$ (by method I; not shown), which is stronger binding than that observed at pH 5.4 ($1 \times 10^3 M^{-1}$; see Table 1).

The results in Table 1 reveal three ranges of binding constants for the trimers studied; 0.5 – 2×10^3 , 4 – 6×10^3 and one sequence, AAG, which gives a value of $12 \times 10^3 M^{-1}$.

Although not all possible trimer sequences have been studied, the results in Table 1 provide insights into the sequence specificity of TMV protein–RNA interactions. Various comparisons are drawn in Table 2, based on the assumption that the binding constants reported are obtained at binding equilibrium, for which there is evidence (see Table 1 legend). A further assumption underlying these comparisons is that all the trimers bind in the same frame on the protein. Although the present experiments yield thermodynamic and not structural information, the reasoning presented below argues against single base recognition as a major determinant of trimer binding, therefore at least compositional isomers such as

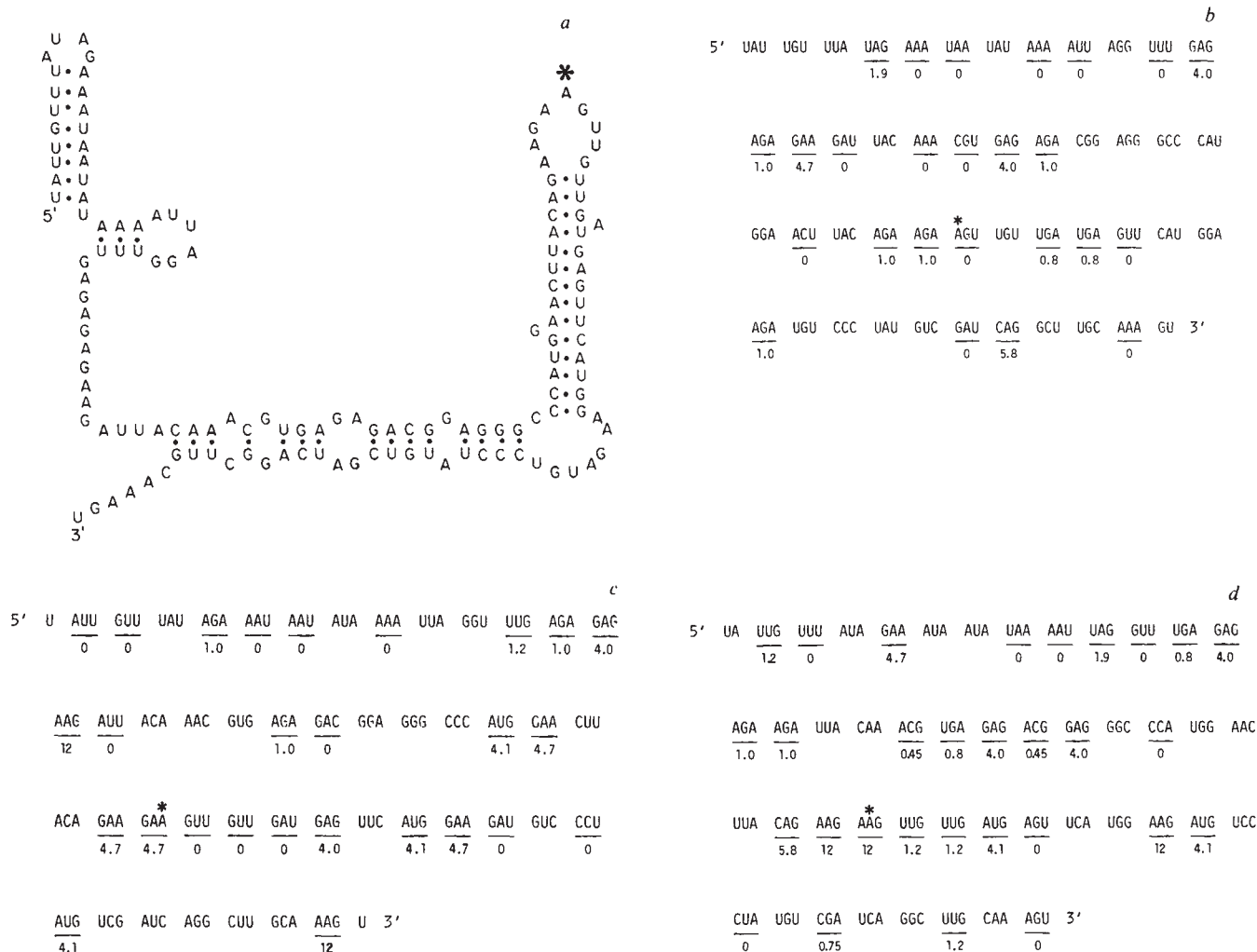


Fig. 2 *a*, Sequence of TMV RNA around the assembly nucleation region, as determined by Zimmermann⁵. Secondary structure is that proposed by Zimmermann. *b*, Alignment of the sequence of trinucleotides at the assembly nucleation site of TMV RNA into a binding frame for TMV protein recognition, as suggested by Zimmermann⁵. Binding constants ($10^{-3} \times K$ (M^{-1})) beneath the underlined trimers are for the corresponding trimers from Table 1. *c*, Trinucleotide binding frame resulting from shifting the frame in *b* one position in the 3' direction. *d*, Trinucleotide binding frame resulting from shifting the frame in *b* one position in the 5' direction.

XXY and YXX would not be expected to bind in different frames.

The comparisons in Table 2 suggest the following general rules for binding. Trimer binding is highly sequence-dependent, in support of Caspar's prediction¹². Guanosine is present in every trimer that binds and appears to lead to the strongest binding when it is in the 3' position. However, an internal pyrimidine inhibits the 3' guanosine contribution to binding, with C having a stronger destabilizing effect than U. The low percentage of C at the assembly nucleation site, relative to its percentage in the whole TMV RNA molecule, has previously been interpreted to mean that C in a trinucleotide sequence exerts a destabilizing effect on binding to TMV protein^{6,24}. The pattern of binding constants in Table 1 does not reveal a simple additive contribution of single bases to the overall free energy of trimer binding, suggesting a cooperative binding mechanism. This can be seen by considering the following comparison in which the alignment of three trimers assumes that single base contributions dominate trimer binding:

It can be seen that each of the protein sites can be occupied identically (neglecting end effects) in the case of the three trimers examined. If single base recognition and binding determine the strength of trimer binding largely in a simple additive manner, the observed binding constants should be almost identical; clearly, this is not the case. Similar comparisons using other examples from the data in Table 1 support the conclusion that single base recognition alone does not account for the trimer binding results. This conclusion is strengthened further if the result that the trimer AAA shows no detectable binding is included in the above comparison.

The correlations described above can be summarized in the following set of rules which accurately describe the binding or non-binding of the 25 trimers tested, and predict the behaviour of the remaining 39.

Three classes of binding trimers ($5' \rightarrow 3'$) have been found: X-Y-guanosine, X-guanosine-purine, and guanosine-X-purine. Thus, the presence of a guanosine in a trimer is necessary but not sufficient for binding as trimers containing a pyrimidine in the $3'$ position do not bind even if there is a guanosine in either the $5'$ or internal position.

A comparison of the free energies of binding for the four XAG trimers, on the assumption that they bind to the protein in the same frame, leads us to conclude that for the base in the 5' position the trimer binding energy may be more sensitive to

		Protein site				Binding constant ($10^{-3} \times K$ (M $^{-1}$))
1	2	3	1	2	3	
A	A	G				12.0
	A	G	A			1.0
		G	A	A		4.7

Table 2 Dependence of apparent binding constants on trimer composition and sequence

<i>a</i>		<i>b</i>		<i>c</i>	
AUG(4.1)		AAG(12.0)		AAG(12.0)	
UAG(1.9)		GAA (4.7)		CAG (5.8)	
UGA(0.8)		AGA (1.0)		GAG (4.0)	
AGU (0)		AAA (0)		UAG (1.9)	
GAU (0)					
<i>d</i>		<i>e</i>		<i>f</i>	
AGA(1.0)		AAG(12.0)		AAG(12.0)	
UGA(0.8)		AUG (4.1)		CAG (5.8)	
CGA(0.75)		ACG (0.45)		AUG (4.1)	
				GAG (4.0)	
				UAG (1.9)	
				UUG (1.2)	
				ACG (0.45)	
					AGA(1.0)
					UGA(0.8)
					CGA(0.75)
<i>g</i>		<i>h</i>			
AAG(12.0) > AAU(0) ≡ AAA (0)		AAG(12.0) > UAG(1.9)			
AUG (4.1) > AUU(0) —		GAA (4.7) > UAA (0)			
GAG (4.0) > GAU(0) < GAA(4.7)		GAG (4.0) > UAG(1.9)			
UUG (1.2) > UUU(0) —		AGA (1.0) ≡ UGA(0.8)			
— AGU(0) < AGA(1.0)		AAG(12.0) > CAG(5.8)			
— GUU(0) —		AGA (1.0) ≡ CGA(0.75)			
ACG (0.45) > ACU(0) —					
— CCU(0) ≡ CCA(0)		Note however:			
— CGU(0) < CGA(0.75)		GAG (4.0) < CAG(5.8)			
GAG (4.0) > GAC(0) < GAA(4.7)					

The data from Table 1 are grouped to test for the dependence of the apparent binding constants ($10^{-3} \times K(M^{-1})$) on trimer composition and sequence. The following conclusions can be drawn from these comparisons: *a*, The binding constant of a trimer depends not on nucleoside composition but on sequence (see first three entries in *b*). *b*, The presence of only purines in a trimer does not necessarily result in binding, which is seen to be strongly sequence-dependent even in a tripurine trimer. *c*, For the trimer XAG the 5' base influences binding in the order A > C > G > U. *d*, In contrast to XAG, the binding of the trimer XGA is much less sensitive to the base in the 5' position. *e*, For the trimer AXG the internal base influences binding in the order A > U > C. *f*, All seven trimers studied which have a G in the 3' position bind. However, a G in the 5' position results in binding only if a purine is in the 3' position. All the 11 trimers which bind have a purine in the 3' position. *g*, None of the 10 trimers studied which have a pyrimidine in the 3' position bind. Ten of the possible corresponding 18 independent 3' purine-substituted trimers were measured and of these, eight exhibited binding, in contrast to the absence of binding in the parent 3' pyrimidine trimers. *h*, In most cases, a 5' purine is necessary for strong binding and substitution for a 5' pyrimidine reduces strong but not weak binding.

the NH₂ and the =O ring substituents than it is to the size of the heterocyclic ring (see Fig. 1).

As the difference in binding energies for 5' pyrimidines, $\Delta(\Delta G^*)_{py} = \Delta G^*(CAG) - \Delta G^*(UAG)$ (where R₁ is =O; see Fig. 1), is about the same as that for 5' purines, $\Delta(\Delta G^*)_{pu} = \Delta G^*(AAG) - \Delta G^*(GAG)$ (where R₁ is NH₂ or H), we assume that these substituents in the R₁ position do not influence binding and the $\Delta(\Delta G^*)$ value of ~ 0.65 kcal mol⁻¹ in both reflects the NH₂ to =O substitution. Continuing on the assumption that binding is insensitive to the R₁ substituent allows a comparison of $\Delta G^*(AAG) - \Delta G^*(CAG)$ and of $\Delta G^*(GAG) - \Delta G^*(UAG)$ where the R₂ positions are the same (NH₂ and =O, respectively). This comparison yields the same value of $\Delta(\Delta G^*)$, that is, ~ -0.45 kcal mol⁻¹, in both cases and suggests that this difference results from the difference in heterocyclic ring size.

These binding properties at the site for the 5' nucleoside contrast strongly with those at the 3' site. From the results for the trimer GAX (see Table 2f, g) it can be seen that the 3' site is more sensitive to heterocyclic ring size than is the 5' site. A detailed stereochemical interpretation of these conclusions must await elucidation by X-ray diffraction of high resolution structures of the oligonucleotide-TMV protein complex²⁵⁻²⁷ and of TMV^{11,28}.

Probable binding frame for nucleation of TMV self-assembly

Figure 2a shows the RNA fragment, sequenced by Zimmer⁵, which is proposed to contain the region for nucleation of *in vitro* virus assembly. The hairpin secondary structure is that proposed by Zimmer based on maximum base pairing. The non-bonded loop of the hairpin structure on the right-hand side (marked *) has been proposed to be the first part of the RNA molecule that binds to the inside of the nucleating disk^{5,7}. The three possible linear binding frames for the interaction of three nucleotides with each of the identical coat protein subunits in either the disk or helix conformation are shown in Fig. 2b-d.

Shown beneath each binding frame are the available binding constants for the corresponding trimers from Table 1. The frame suggested by Zimmer⁵ is shown in Fig. 2b. Figure 2c gives the trimer binding frame shifted one position in the 3' direction from Fig. 2b. The 5'-shifted reading frame is shown in Fig. 2d. A comparison of the available trimer binding constants in Table 1 with the three binding frames reveals that the trimer alignment centred around AAG (Fig. 2d) results in the greatest number of tight sites for protein binding.

Binding frame *d* contains the greatest number of strong binding sites and the largest total number of binding sites. Comparison of the number of trimer binding sites in the three binding frames shows that frames *b*, *c* and *d* have, respectively, 4, 11 and 10 binding sites with trimer affinity constants equal to or greater than 4×10^3 M⁻¹. A more significant comparison which distinguishes between binding frames *c* and *d* is the presence of three 12×10^3 M⁻¹ trimers in the latter and two in the former. In binding frame *d* two of the highest affinity trimers, AAG, are contiguous whereas in *c* the two AAG sequences are widely separated. To a first approximation it would be expected that trimers in such pairs contribute to binding as the product of the individual trimer binding constants. This would mean that only binding frame *d* contains a region having an affinity $\geq 10^8$ M⁻¹. The sequence forming this region, —AAG—AAG—, is in the large loop region (marked * in Fig. 2a) thought to be involved in the initial binding by the disk protein aggregate^{5,7}. This loop is flanked by other trimer sequences, CAG and UUG, which bind and would thus further enhance the stability of the initial complex by ~ 5 kcal beyond the ~ 11 kcal provided by the —AAG—AAG— sequence. In general, summation of trimer binding free energies, in order to estimate free energies for larger oligonucleotides, implicitly assumes that the trimers all bind to the helical protein rods in the same binding frame. However, such an assumption is obviously not required for deriving the ~ 11 kcal provided by the hexamer —AAG—AAG— but is necessary when adding the binding energies of the adjacent trimer sequences, CAG and UUG, in the RNA loop region marked (*) in

Fig. 2a. Binding energies of at least ~ 15 kcal are probably necessary for highly specific protein-nucleic acid interactions. Co-operative binding effects between adjacent trimer sites would contribute further to the binding energy for nucleation. To estimate the minimum energy of the RNA-protein interactions, we assumed that the helical rods of TMV protein at $\text{pH} < 6$ are, to a first approximation, good representatives of TMV without the RNA^{15,19,20}. There are, however, differences in protein structure between the helical protein rods and TMV^{19,20}. Studies of the energetics of changing the protein structure in the RNA-free helical rods to that occurring in TMV in conditions of *in vitro* TMV assembly are required to define fully the energy of interaction between a trinucleotide and the RNA binding site in the final virus structure. However, the energy of this conformational change is expected to be small because the titration curves of the whole virus and the RNA-free helical protein rods are almost identical, differing by at most 0.5 kcal per mole of subunits¹⁵. Evaluation of the total free energy of assembly nucleation at $\text{pH} 7.0$ will require knowledge of the energetics of the switching of the protein conformation (disk $\rightleftharpoons$ helix). Although there are no accurate values for the free energy of the reaction (disk $\rightleftharpoons$ helix), one can estimate a value between 2 and 4 kcal per mole of subunits from an analysis of titration¹²⁻¹⁷ and sedimentation data⁹. The upper range of this estimate is consistent with the observed very weak binding of the AAG trimer to 20S protein which is thought to be mainly the disk aggregate at $\text{pH} 7$.

The alternative hairpin-loop structure of the nucleation region of TMV RNA proposed by Jonard *et al.*⁶ is not expected to alter the conclusion regarding the RNA binding frame for protein binding. An inspection of their structure shows that the —AAG—AAG— hexanucleotide is located at the top of the loop, and therefore is in a position on the RNA to interact with the initiating 20S protein aggregate in much the same way as is the same hexanucleotide of Fig. 2d.

Discussion

These binding experiments were done in conditions that ensured a very low fractional site occupancy to avoid the complication of trimer binding to possible overlapping sites in the helical protein rods. We assume that the trimers bind to the tightest sites available to them and that they bind in an orientation similar to that of the natural RNA in the virus. There is no way of knowing from these studies alone whether the trimers were bound directly to the trinucleotide binding site of the individual protein subunits or in a site formed by two laterally adjacent subunits in the helical rods. However, based on a recent refinement of the 4 Å fibre diffraction pattern of TMV and the suggested structure of the RNA in the virus²⁸, we consider that inter-subunit trinucleotide binding would not be favoured in the conditions of our experiments. The sequence dependence of the binding affinity of trinucleoside diphosphates to TMV protein suggests that the RNA binding frame of

Fig. 2d is probably the one that promotes binding to the initiating TMV protein aggregate.

The results of the binding studies described here may provide only part of the explanation for nucleation of TMV self-assembly occurring at the region of the TMV RNA molecule shown in Fig. 2d, as previous studies have shown that small fragments of TMV RNA derived from parts of the molecule other than the assembly nucleation region bind to disk-like aggregates of TMV protein^{24,29}. These results have been interpreted to mean that the specificity of nucleation is defined by an RNA sequence that, once bound, promotes the quaternary conformational change of the initiating two-turn cylindrical disk to a two-turn helix⁵. However, the results reported here are consistent with the trimers binding selectively to subunits in a helical array in the (disk $\rightleftharpoons$ helix) equilibrium.

Binding of fragments of TMV RNA can be termed non-specific if there is no concomitant switching of the protein conformation. It remains to be demonstrated whether the binding of the RNA fragments derived from the coat protein cistron of TMV RNA²⁴ (the cistron starts ~ 150 nucleotides from the 3' end of the assembly nucleation region³⁰) to aggregates of TMV protein results in the formation of nucleoprotein complexes having a helical structure⁶. In the three binding frames of the largest RNA fragment, T1, there are no regions of sequential binding trimers, based on the information in Table 1, that contain contiguous high-affinity AAG trimers. Tyulkina *et al.*²⁹ have shown that some of the 30S complexes formed by combining 3S fragments of TMV RNA with a preparation of TMVP containing 20S aggregates have a helical structure similar to that of TMV. Their results²⁹ and the demonstration that the T1 and P1 RNA fragments (the P1 fragment contains the assembly nucleation site sequence⁶) bind with comparable rates to the disk aggregate⁵ suggest that the secondary and tertiary structures in the TMV RNA molecule might contribute to the efficiency of the sequence specificity reported here. Certainly, the existence of higher orders of RNA structure does not sufficiently explain the sequence specificity of trinucleoside diphosphate binding to TMV protein. However, RNA folding may control the accessibility of different regions of the RNA molecule to the 20S and 4S aggregates of TMV protein. For example, it is important to know whether nonspecific binding of TMV protein to the RNA molecule is minimized by placing favourably recognized oligonucleotide sequences in the hydrogen-bonded stem regions of hairpin structures rather than in their single-stranded loops. We are confident that the sequence specificity seen for trimer binding to TMV protein is related to the specificity of nucleation as a sufficiently high concentration of AAG causes crystals of the cylindrical disk protein aggregate to dissolve²⁵. This finding suggests that the AAG trimer binds like natural TMV RNA and promotes the disk-to-helix quaternary conformational change²⁶.

We thank Dr David A. Yphantis for suggestions and discussions. This work was supported by NIH grant AI 11573.

Received 27 January; accepted 24 June 1982.

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LETTERS TO NATURE

Galaxy formation by dissipationless particles heavier than neutrinos

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In a baryon dominated universe, there is no scale length corresponding to the masses of galaxies. If neutrinos with mass < 50 eV dominate the present mass density of the universe, then their Jeans mass $M_{J\nu} \sim 10^{16} M_\odot$, which resembles super-cluster rather than galactic masses. Neutral particles that interact much more weakly than neutrinos would decouple much earlier, have a smaller number density today, and consequently could have a mass > 50 eV without exceeding the observational mass density limit. A candidate particle is the gravitino, the spin 3/2 supersymmetric partner of the graviton, which has been shown¹ to have a mass ≤ 1 keV if stable². The Jeans mass for a 1-keV noninteracting particle is $\sim 10^{12} M_\odot$, about the mass of a typical spiral galaxy including the nonluminous halo. We suggest here that the gravitino dominated universe can produce galaxies by gravitational instability while avoiding several observational difficulties associated with the neutrino dominated universe.

There is evidence that a considerable fraction of the mass in the Universe is nonbaryonic. Light element synthesis in the big bang requires that the density parameter³ $\Omega_b = \rho_b / \rho_{\text{crit}} < 0.014 H^{-2}$, where ρ_b is the present baryonic density, $\rho_{\text{crit}} = 11 \text{ keV cm}^{-3} H^{-2}$ is the closure density of the universe, and H is the Hubble parameter in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$ (observationally, $0.4 < H < 1$).

On the other hand, observations give a lower bound⁴⁻⁶ on the total density parameter $\Omega > 0.1$. On a galactic scale, there is also strong evidence that the mass in stars is considerably less than the mass of the nonluminous halo.

The present neutrino number density is $109 \text{ cm}^{-3} (T/2.7)^3$ per species. Because observationally the density parameter Ω cannot significantly exceed unity, the sum of the masses of all stable neutrino species is < 100 eV. Experimentally, the electron neutrino mass is < 50 eV, with one experiment indicating a non-zero mass⁷. Particles such as gravitinos which interact much more weakly than neutrinos decouple at a temperature T_d much higher than the neutrino decoupling temperature and long before many species have annihilated and heated (thereby increasing the number density of) the remaining interacting particles (including neutrinos). For $\Omega H^2 < 1$, the resulting upper limit on the mass of such particles (assuming two interacting helicity states) is $19g(T_d) \text{ eV}$, where $g(T)$ is the effective number of interacting species including neutrinos. The standard particle physics model (QCD plus Weinberg-Salam with three quark and lepton generations) gives $g = 50$ above the quark deconfinement temperature of a few hundred MeV, growing slowly to $g = 100$ at $T \sim 100$ GeV, with perhaps another factor of two growth due to supersymmetry. Therefore any such particle must be less massive than ~ 1 keV. Here we consider the possibility that such particles dominate the Universe and, for definiteness, assume them to be gravitinos of mass ~ 1 keV.

The scenarios for galaxy formation strongly depend on whether baryonic matter, neutrinos or gravitinos dominate the present Universe (see Fig. 1). Baryon condensations of galactic size are strongly damped during the radiation era by Compton drag (Silk damping)^{8,9}. For non-interacting particles of mass m ,

the maximum Jeans mass is given by^{10,11} $M_J \sim (M_{\text{Planck}})^3 / m^2$, which, for Friedmann cosmologies, is the mass within the horizon when the particles become nonrelativistic. The free streaming of dissipationless particles such as neutrinos or gravitinos destroys density perturbations on all scales smaller than the horizon until the particles become nonrelativistic. In particular, the proper free streaming distance travelled by a noninteracting particle with present momentum p_0 between time t and the present time t_0 is¹²

$$D(p_0, t) = R_0 \int_t^{t_0} dt v(t) / R(t)$$

where $R(t)$ is the scale factor of the universe and $v(t)$ is the instantaneous velocity of the particle with respect to a co-moving frame. We take the Jeans mass at time t to be the present mass of particles within a sphere of radius $D(p_0, t)$, with p_0 the characteristic momentum at the present time (ref. 12, appendix).

The most important aspect of Fig. 1 is that for gravitinos the maximum Jeans mass $M_{Jg} \sim 10^{12} M_\odot$ while for neutrinos $M_{J\nu} \sim 10^{16} M_\odot$. Therefore gravitinos provide a natural scale size for galaxies, while the neutrino-dominated universe can form galaxies only by fragmentation of much larger gravitational condensations^{13,14}.

The linear growth of neutrino and gravitino perturbations, of masses $10^{16} M_\odot$ and $10^{12} M_\odot$ respectively, is illustrated in Fig. 2. The density contrast $\delta = \delta n / n$ is a gauge dependent quantity, and here we follow the standard treatment^{15,16} in time-orthogonal coordinates. Adiabatic perturbations larger than the horizon grow as $\delta \propto R^2$ in a radiation-dominated universe, while $\delta \propto R$ for nonrelativistic particles in a matter-dominated universe. Before matter (gravitino) domination, nonrelativistic gravitino perturbations with $M > M_{Jg}$ but smaller than the horizon can grow by at most a factor 2.5 (refs 17, 18), since once inside the horizon radiation oscillates and relativistic neutrinos free stream away. Thus limited growth in δ occurs for $M_{Jg} < M < M_{\text{eq}}$ = horizon mass when matter domination first occurs $\approx 10^{16} (\Omega H^2)^{-2} M_\odot$.

If the initial perturbation spectrum is $\delta(M) \propto M^{-(3+n)/6}$, then in the matter dominated era $\delta(M)$ falls off steeply for $M < M_{Jg}$ due to free streaming of the relativistic gravitinos, is flattened with $n_{\text{eff}} \approx n - 4$ because of the effect just discussed for $M_{Jg} < M < M_{\text{eq}}$, and reflects the initial spectrum for $M > M_{\text{eq}}$. (This has been calculated in detail using the Boltzmann equation by

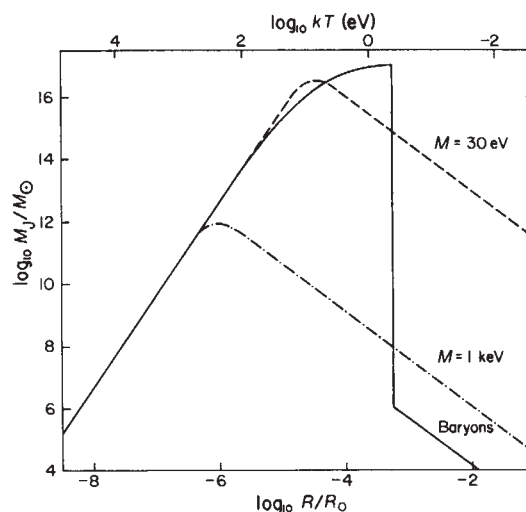


Fig. 1 The Jeans mass versus scale factor for a baryon, gravitino ($m = 1$ keV), and neutrino ($m = 30$ eV) dominated universe with $\Omega H^2 = 1$. Neutrino and gravitino perturbations with $M < M_J$ at any time are dissipated by free streaming.

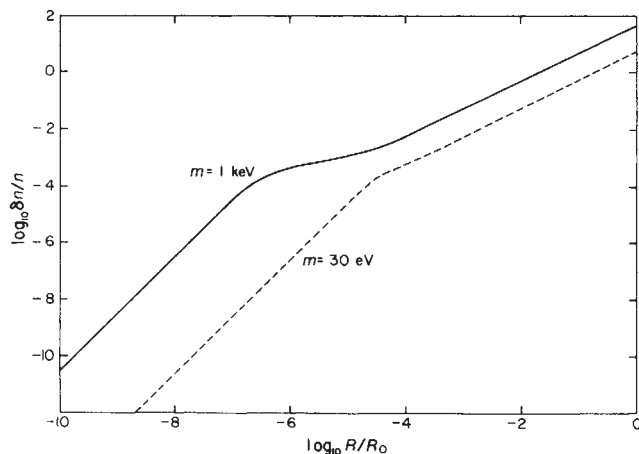


Fig. 2 The growth of linear perturbations on mass scales $> M_J$ versus the scale factor of the universe ($\Omega H^2 = 1$) for noninteracting particles of mass 1 keV and 30 eV. The overall normalizations of these curves are arbitrary.

Bond *et al.*¹⁹.) Finally, after hydrogen recombination, baryon fluctuations rapidly grow towards those of the gravitinos²⁰. Consequently, in the gravitino case, an initial adiabatic perturbation spectrum with $n \approx 1$ leads to the formation of galaxies of total mass $\sim 10^{12} M_\odot$, followed by baryon dissipation on galactic scales only²¹ (consistent with cooling constraints²²), with hierarchical clustering on scales between $\sim 10^{12}$ and $\sim 10^{16} M_\odot$. Observationally²²⁻²⁴, there is little variation in matter density from small clusters to superclusters, which in the absence of dissipation implies that clusters of all sizes collapsed roughly simultaneously—that δ is nearly constant between 10^{12} and $10^{16} M_\odot$. Such a spectrum ($n_{\text{eff}} \approx -2$) follows in the gravitino scenario from the plausible initial spectrum $n \approx 1-2$. Also, in contrast to the neutrino scenario where the formation of superclusters through dissipational pancake collapse^{13,14} precedes the formation of galaxies and must therefore have occurred at $z > 3$, in the gravitino scenario supercluster collapse follows galaxy formation and is dissipationless. This is consistent²⁵ with the observed²⁶ absence of clustering of Ly α absorption lines in quasar spectra and with the lack of extreme flattening in superclusters²⁷.

The nonluminous haloes of galaxies may also distinguish between the neutrino and gravitino hypotheses. If large spiral galaxies such as our own have haloes with radius ~ 100 kpc and $M/L \approx 50$, then there is not more than about a factor of two growth in the total/baryonic mass ratio from galactic to rich cluster scales. This is expected in the gravitino picture but not in the neutrino picture, where only $\sim 10\%$ of neutrinos are cooled enough to be captured by galaxies (A. Melott, and J. R. Bond, personal communications). It has indeed been suggested recently that the data are consistent with an approximately constant ratio of 7% baryonic to total mass on all scales larger than ~ 100 kpc (refs 22, 28). Finally, the phase space constraint^{29,30} on the mass of a fermionic halo particle is strongest for dwarf galaxies, for which preliminary analysis (S. Faber and D. Lin, in preparation) suggests this mass is at least several hundred eV.

We thank J. R. Bond, M. Davis, R. Giles, W. Mathews, A. Szalay, and especially S. Faber and D. Lin for helpful discussions, and the NSF and DOE for partial support. This is Lick Observatory Bulletin no. 931.

Received 5 January; accepted 15 July 1982.

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Discovery of 0.5-s oscillations in long flat top bursts from the Rapid Burster

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We report here a search for periodic emission of X rays from MXB1730-335 (the Rapid Burster, which is the most unusual X-ray burst source¹ out of about 30 other bursters) during the long flat top bursts². Two events from the 63 bursts showed clear peaks in the frequency power spectra at ~ 0.5 s in period. However, this is unlikely to be a pulsar period, because the periods of these two bursts are different.

The burst activity of the Rapid Burster was observed between 8 and 22 August 1979 with the X-ray astronomy satellite Hakucho. From 8.29 to 15.97 August (UT), bursts were exclusively long flat top ones. Owing to their high intensities and long durations, we were able to search for the periodicity of the X-ray intensity during these events.

We analysed 63 long flat top bursts of durations in the range 40-600 s. The power spectrum analysis was performed for the bin width of 93.75 ms, the shortest time bin available, over the frequency range 0.1-5.3 Hz. All but two bursts show no periodicity higher than statistical fluctuations. The upper limits of pulsed fractions thus obtained are 10% of the intensity during the flat tops of the bursts.

For the two long flat top bursts which occurred at A 19 h 04 min on 9 August and B 01 h 45 min on 11 August (UT), oscillations were discovered with the mean periods of 0.508 ± 0.001 s for burst A and 0.503 ± 0.001 s for burst B. Periods were determined both by the power spectrum analysis and by the folding analysis. Figure 1 shows the time profiles and the power spectra of these two events. The power spectra show clear peaks with the maximum power density of 23-24 times the average power density level of white noise, corresponding to a pulsed fraction of 30%. Statistical errors of these periods were estimated by the folding analysis. If we calculate the χ^2 defined as

$$\chi^2 = \sum_{k=1}^n (f_k(P) - \bar{f})^2 / s_k^2$$

where $f_k(p)$ is k th bin of the pulse profile folded with a trial period p , $\bar{f}$ is the average of $f_k(p)$ over n total bins and s_k^2 is a variance of each bin, then a real period P_0 corresponds to the maximum χ^2 ; $\chi^2(P_0) = \chi^2_{\text{max}}$. A statistical error of period

Table 1 Characteristics of two long flat top bursts showing 0.5-s oscillations

	A 9 August 19 h 04 min	B 11 August 1 h 45 min	C* 8–22 August
Burst			
Duration (s)	123	150	5–600
Peak luminosity† (erg s ⁻¹)	$(3.27 \pm 0.09) \times 10^{38}$	$(3.72 \pm 0.08) \times 10^{38}$	$(0.7–4.0) \times 10^{38}$
Total energy† (erg)	$(4.20 \pm 0.10) \times 10^{40}$	$(5.55 \pm 0.12) \times 10^{40}$	$(0.1–8.0) \times 10^{40}$
Black-body temperature (keV)	1.97 ± 0.06	2.00 ± 0.03	1.5–2.0
Oscillations			
Mean period (s)	$0.508 \pm 0.001\ddagger$	$0.503 \pm 0.001\ddagger$	
Range of period (s)§	0.50–0.52	0.50–0.52	
Mean pulsed fraction	0.28 ± 0.05	0.30 ± 0.05	
Maximum pulsed fraction in 24 s	0.52 ± 0.10	0.40 ± 0.10	

* The ranges of burst parameters for the total data.

† Assuming the spherical emission at a distance of 10 kpc.

‡ Statistical errors estimated by the folding analysis, see text.

§ Obtained from dynamical power spectra for 24 s.

ΔP is estimated as follows:

$$\chi^2(P_0 \pm \Delta P) = \chi^2_{\max} - 1$$

The possibilities that these oscillations are of instrumental or geophysical origin can be excluded by the following tests. (1) The period does not correspond to instrumental time constants and their harmonics that would cause the observed power spectrum peak. (2) Oscillations with a period near 0.5 s are not found in the background data of 1.8 h obtained during this period of active bursts. (3) The occurrence of the present oscillations have no correlation with geographical position and local time of the satellite. We therefore conclude that the observed oscillations are in the X-ray flux.

Characteristic properties of these two bursts and of the oscillations are summarized in Table 1. These two bursts occurred during the highest burst activity; the bursts during this period were of largest size, highest peak luminosity and had the hardest spectra¹. There are many bursts similar to the

oscillating events in size, luminosity and duration, but they show no oscillations. None of the 15 bursts observed between the two oscillating events A and B shows periodicity.

Several interesting properties can be noted for the observed oscillations. Figure 2 shows the power spectra of these two bursts every 24 s, where the statistical uncertainties of periods ΔP are 2 ms. Both a drift of the frequency and a variation of the pulsed fraction are clearly seen. For event A, the period of the maximum power density appears to have moved from 0.50 s to 0.52 s, the pulsed fraction increasing up to $40 \pm 10\%$, and the oscillation disappearing at ~ 20 s before the start of decay of the burst. Similar characteristics are observed for the other burst B as shown in Fig. 2.

Figure 3 shows the pulse profiles for energy band $L(1–9$ keV) and $H(9–22$ keV) respectively, obtained by folding the data of Fig. 2 *Ab* and *Bc* with periods of 0.511 s and 0.500 s for events A and B, respectively. The hardness ratios of the burst at the peak and the bottom of the folded profile are 0.296 ± 0.032 , 0.278 ± 0.046 respectively, thus they are the same within the

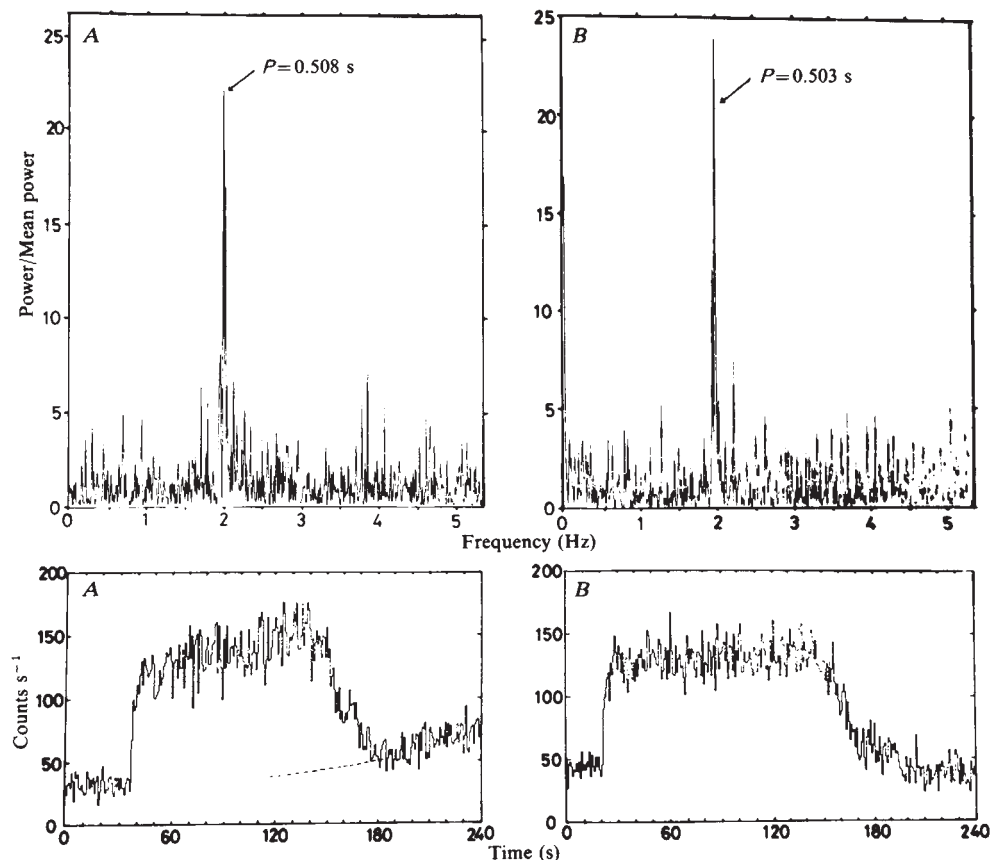


Fig. 1 The time profiles and the power spectra of the two long flat top bursts of 0.5 s oscillations. The time profiles start at A, 19 h 03 min 30 s on 9 August and B, 1 h 45 min 00 s on 11 August (UT) in 1979. The dashed line in the time profile of event A shows an increasing background level.

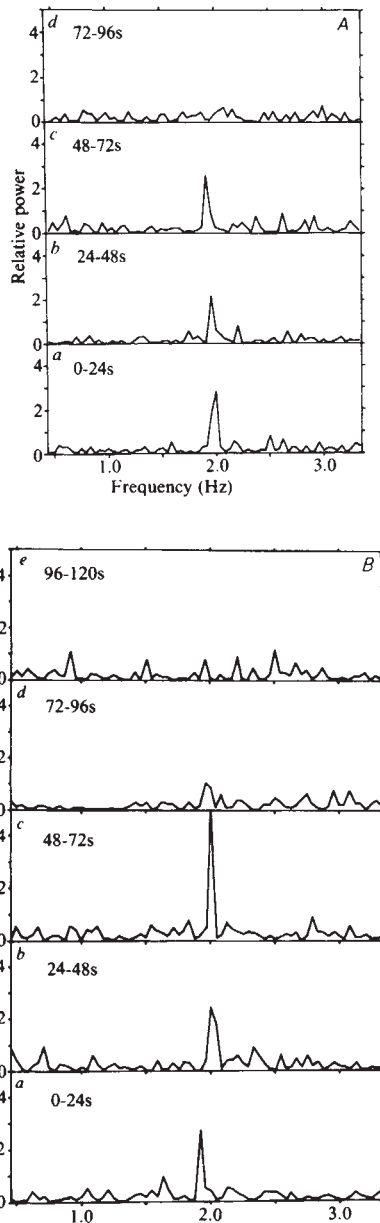


Fig. 2 The power spectra of events *A* and *B* for every 24 s, showing a drift of the period and/or phase of oscillations. Times indicated in each power spectrum in seconds are from 19 h 04 min 10 s on 9 August and 1 h 45 min 38 s on 11 August (UT), respectively.

error limits. A nearly sinusoidal pulse profile is expected from the fact that the first higher harmonics is not significant in the power spectra shown in Fig. 1.

As the 'rapid' bursts have been considered to be indicative of gravitational energy release by the accreting matter onto a neutron star³, the following two time scales of periodicity, within which the X-ray emission may be modulated, are conceivable: (1) a stellar rotational period; (2) a keplerian period of matter orbiting around the neutron star. A 0.5-s period is an acceptable value in a pulsar period. However, a 1% change of the mean period for the two bursts separated by ~ 31 h, is too large to be accounted for by the rotating neutron star. The rotator model is also excluded by the facts that only two bursts show pulsations, that the oscillations disappear towards the end of the bursts, and that the period drifts during the burst.

The second possibility is related to a model proposed for the periodic oscillations observed in optical and X rays during outbursts from several dwarf novae such as SS Cyg (refs 4, 5).

The oscillations which are considered to take place in viscous accretion disks⁶ may also occur in an accreting neutron star with a period equal to the keplerian period at the magnetopause and then modulate the rate of accretion from the accretion disk to the neutron star. The radius of keplerian orbit with the period of 0.5 s is $\sim 10^8$ cm for a neutron star with the mass of $\sim 1 M_{\odot}$ and corresponds to the radius of the magnetopause for a magnetized neutron star with a magnetic field of $\sim 10^{12}$ G.

It is therefore conceivable that the modulation with a 0.5 s period in the matter supply from the region near the mag-

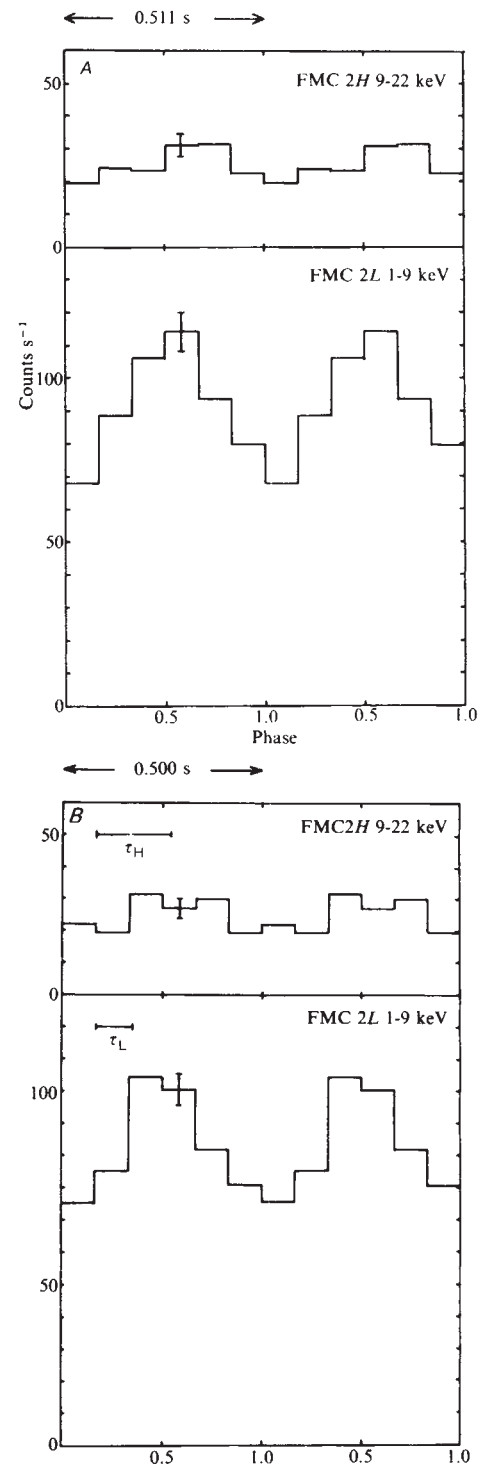


Fig. 3 The time profiles, folding the data shown in Fig. 2 *A*, *b* and *B*, *c* with periods of 0.511 s and 0.500 s, separately for *L*(1–9 keV) and *H*(9–22 keV) bands. The time, τ_L and τ_H , shown represents the data sampling time for the *L* and *H* bands, respectively.

netopause to the surface of the neutron star results in the observed oscillations of the rapid burster. The fact that the hardness ratios of the bursts at the maximum and the minimum of the pulse are the same supports this possibility.

We thank the crew of the Hakucho satellite for providing observational data and to Professors Y. Tanaka and M. Matsuoka for their valuable comments.

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A possible resurfacing mechanism for icy satellites

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Photographs from Voyager Missions reveal a great variety of surface features for the icy satellites of Saturn¹. Mimas is covered with many old craters. Enceladus has a rather smooth surface with few and rather young craters. Some parts of Dione's surface have an important crater density, whereas other parts are relatively smooth. Thus some of Saturn's icy satellites seem to have undergone important surface modifications during their history while others did not. I suggest here that the irreversible phase transition between amorphous and cubic ice and similar phase transitions may provide a low energy resurfacing mechanism for these icy satellites.

To explain the resurfacing of Enceladus it has been suggested² that its interior may contain liquid water covered only by a thin crust of ice. For a given thickness of the crust, it is possible to estimate what the subsurface heat flux must be. For a surface temperature of 100 K, a liquid core temperature of 273 K and a heat conduction coefficient like that of normal hexagonal ice³ we obtain a heat flux in the order of magnitude of 0.1 W m^{-2} . This is comparable to the mean terrestrial heat flux ($\sim 0.063 \text{ W m}^{-2}$). If this heat flux has to be maintained permanently by tidal heating, the tidal dissipation must be at least $7 \times 10^{10} \text{ W}$ in the whole satellite. Peale *et al.*⁴ calculated the average volumetric tidal dissipation in Saturn's satellites. They found for Enceladus $2.2 \times 10^{-10} \text{ W m}^{-3}$. That corresponds to a total dissipation of $1.4 \times 10^7 \text{ W}$. So it seems difficult to maintain a liquid core by tidal heating and even more difficult to heat up a low temperature core and to melt it.

I now propose an alternative resurfacing mechanism that needs much less initial energy input. It has been shown^{3,5,6} that the irreversible phase transition of water ice that occurs between amorphous and cubic ice at $\sim 153 \text{ K}$ may influence the heat balance of comet nuclei. The same phase transition may in some cases be responsible for resurfacing of icy satellites. Suppose that the satellite has been condensed at temperatures sufficiently low so that the ice is in the amorphous state. As amorphous ice is a very poor heat conductor, we can assume that a large part of the heat dissipated in the interior of the icy body is used to heat it up. When in one part of the satellite the transition temperature is reached, the phase transition will start. The heat release during crystallization is about 100 J g^{-1} (ref. 7). This energy can be used to heat the neighbourhood. On the other hand the heat conduction coefficient of crystalline ice at the transition temperature seems to be about 10 times higher than that of amorphous ice³ and a growing part of the internally dissipated heat will be lost to the surface. These two mechanisms

are in competition. But as the crystallization of the amorphous ice will start in the interior the heat loss will be unimportant until the surface layers undergo the phase transition.

In the case of Enceladus the tidal dissipation calculated by Peale *et al.*⁴ is able to heat this body from 100 to 153 K in 7,000 Myr. This is evidently not realistic. But Ghormley⁷ found that amorphous ice condensed at 20 K and released 33 W g^{-1} during heating from 20 to 77 K and 92 W g^{-1} between 77 and 153 K. The heat release is smaller for ice condensed at higher temperatures. This heat will considerably speed up the process and only slight heating by tidal dissipation is necessary to initiate the temperature rise.

On the other hand, Yoder⁸ proposed that the eccentricity of Enceladus has been pumped to a higher value than the present one. This may produce some excess heating and allow a still more rapid heating from the orbital equilibrium temperature to the phase transition temperature. Evidently the mechanism described here may be combined with resurfacing mechanisms of very different nature.

Amorphous ice has a density between 1.1 and 0.94 g cm^{-3} depending on the condensation temperature⁵. During the phase transition nearly all water molecules are reoriented and the density decreases. This decrease in density may produce surface cracks and contribute to the smoothing of existing surface features.

If the heat transport to the surface due to conduction is not able to evacuate all the heat dissipated in the icy body, the internal temperature will continue to rise and might reach the transition temperature between cubic and hexagonal ice where a second resurfacing process may take place. A third resurfacing may take place when the melting temperature of hexagonal ice can be reached. When the heat loss to the surface becomes more important than the internal dissipation the satellite will cool down to the equilibrium temperature; in the case of Saturn's satellites to a temperature near 100 K. This cooling can take place after a partial or a complete resurfacing or after one or more resurfacing periods.

It is evident that similar mechanisms may apply when other materials undergo similar phase transitions. We know, for example, that the heat conduction coefficient of at least one clathrate hydrate is of the same order of magnitude and has a similar temperature dependence as amorphous substances⁹. We therefore suppose that such kind of effects will appear when clathrate hydrates reach their limits of stability.

According to Gaffney and Matson¹⁰, high pressure forms of ice may locally be produced during crater formation. Those forms are metastable at low pressures for temperatures that can be found on Jupiter's and Saturn's satellites. The lifetime of those polymorphs is evidently temperature dependent. Destruction of those ices may also contribute to a resurfacing. Gaffney and Matson suggest that icy satellites should be investigated by means of IR reflection spectroscopy. In fact, morphological studies of impact crater and other surface features combined with IR scanning reflection spectroscopy during future space missions could yield information about the chemical nature and the physical state of surface materials, on the chronology of surface smoothing, and the mechanisms involved. Those kinds of mechanism may occur on many satellites of the outer planets.

More quantitative work on the problem of the geological activity of icy satellites is planned in our laboratory. This work has been sponsored by INAG-ATP Planetologie grant 37-86.

Received 5 April; accepted 23 June 1982.

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Flare induced geomagnetic activity and orientation of the photospheric magnetic field

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Attempts to determine which solar flares produce geomagnetic storms have focused attention on the importance of the direction of the magnetic field at the flare sites. Pudovkin *et al.*¹⁻³ have claimed that solar flares that occur above regions in which the photospheric magnetic fields have southward components produce geomagnetic storms whilst flares associated with northward magnetic fields do not. However, other studies have shown that geomagnetic storms are also highly correlated with energetic flares⁴, especially those accompanied by radio spectral type IV bursts⁵⁻⁸. McNamara⁹ suggested that these two sets of results were not reconcilable because they implied that nearly all type IV bursts were associated with flares with southward fields. Although this implication seemed unreasonable, recent evidence^{9,10} lends it some support. We have investigated the relationships between geomagnetic disturbances, flare energies and flare-site magnetic field directions for all large flares that occurred between 1968 and 1979. Contrary to the results of Dodson *et al.*¹⁰ we find no significant differences between the average energies of flares with northward, southward or east-west magnetic fields. Furthermore, although the inferred direction of the field at the site of a flare appears to control the geoefficiency of the flare to some extent, we find, contrary to Pudovkin *et al.*¹, that the relationship between geoefficiency and field direction is unclear.

Our study included 342 flares (with longitudes $\leq 70^\circ$) of importance 2 or greater that occurred between 1968 and 1979¹¹. Using the Mt Wilson and Kitt Peak magnetograms¹², we attempted to determine the photospheric magnetic field direction underlying the centroid of each flare under the assumption that the field was directed at right angles to the contours of magnetic intensity. Our procedure for inferring the field direction underlying the flare follows that of Pudovkin *et al.*¹. However, we make no assumptions concerning the relation between this direction and the true direction of the magnetic field within the flare site which lies above the photosphere in the chromosphere and base of the corona¹³. (Hereafter, unless otherwise stated, it is the inferred direction of the photospheric field that is implied in all discussion of the magnetic field direction at the flare site.) In many cases the flare centroid was located above strongly curved contours and it was not possible to ascribe an accurate field direction. For this reason we categorized the directions into eight broad ranges (45° wide) centred on the eight cardinal directions N, NE, E, SE, S, SW, W and NW. Thus our field directions were defined with respect to the heliographic meridian of longitude at the flare site whereas Pudovkin *et al.*¹ defined their directions with respect to the projected orientation of the Earth's axis. We have compared our field directions with those determined by Pudovkin *et al.*¹ for all flares that were common to both studies and we found good agreement. In particular, nearly all flares that had northward and southward field components with respect to the Earth also had northward and southward components (respectively)

Table 1 Distribution of flares by field direction

N	NE	E	SE	S	SW	W	NW
22	33	34	35	27	17	34	35

Field direction uncertain, 72. No magnetic data, 33. Total, 342.

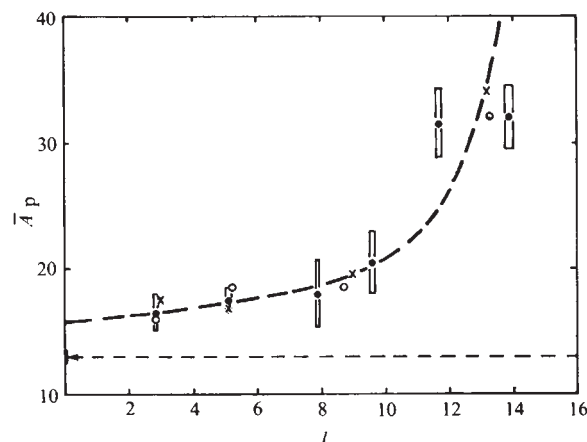


Fig. 1 The dependence of geomagnetic activity on the flare energy index. Each flare in our sample was grouped according to the magnitude of its energy index, I . The average value ($\bar{A}_p$) of the geomagnetic index, A_p , on the second and third days after the flares in each group was calculated. $\bar{A}_p$ was then plotted against the average value of I for each group. The results for our entire sample of flares ($\bullet$) are shown for which the magnitudes of the standard errors of the means are indicated by boxes. Also shown are the results (without error boxes) for flares in the periods 1969-76 ($\circ$) and 1977-79 ($\times$). The mean background level of A_p is indicated by the horizontal broken line.

with respect to the Sun. Table 1 shows the distribution of the flares amongst the eight cardinal directions.

To estimate the relative energies of the flares in our sample we conceived a flare energy index, I , which is similar to the 'comprehensive flare index' (CFI) proposed by Dodson-Prince *et al.*⁴. We have introduced our own index because it is directly calculable from data to which we have ready access whereas the CFI is not. Our index takes into account flare characteristics that indicate high energy release^{13,14}, particularly those found by McNamara⁹ to be important in the flare-storm relationship. These were H α importance and intensity, X-ray class and radio spectral class. To each characteristic we attached a numerical weight in the manner shown in Table 2 and the value of the index for a given flare was then determined by summing the individual weights. Hence the least important flare in our sample, a 2F event with no radio or X-ray bursts, corresponds to a flare index of $I = 2$. The most important flare in our sample, a 3B event with X-class X-ray-ray burst, type II and strong type IV bursts, corresponds to an index $I = 15$. No X-ray data was available for 1968 so that I could not be computed for flares in that year. Also, there were a few flares in subsequent years for which I could not be computed due to incomplete data.

The level of geomagnetic activity following the flares in our sample was assessed by means of the method of superposed epochs which we applied to the daily geomagnetic index A_p . In agreement with Dodson-Prince *et al.*⁴ we found that A_p usually peaked 2-3 days after large flares. Hence to assess the geomagnetic importance of flares within any particular class we used the average value of A_p ($\bar{A}_p$) on the second and third days after the flares. The dependence of $\bar{A}_p$ on the energy index, I , is shown in Fig. 1. The average level of geomagnetic activity increases slowly with I for flares with energy indices in the range $0 < I \leq 10$. However, when $I > 10$ the average level of geomagnetic activity increases rapidly with I . This is consistent with studies^{4,8} that show that very large flares nearly always produce geomagnetic storms. Figure 1 also shows that I preserves its identity over a long period of time since data for 1969-76 and 1977-79, when considered separately, define the same relationship between I and A_p . Therefore, we consider I to be a useful index with which to describe, semiquantitatively, the relative energy release of large solar flares.

Using the calculated values of I for all the flares in our sample, we were able to investigate the relative contributions

of flare energy release and magnetic field direction in the flare-storm relationship.

First, we considered whether a relationship existed between energy release and flare-site field direction. For this purpose we calculated the average values of I for flares with northward, southward and east-west magnetic fields. The results are shown in Table 3. In addition to the flares in our sample we have considered the flares listed by Pudovkin *et al.*¹. Several cases are distinguished. Case 1 includes all flares described by Pudovkin *et al.*¹ for which I could be determined. All but three of these flares occurred between 1969 and 1970. In this case the subdivision of flares amongst field directions was made using the directions measured by Pudovkin *et al.*¹. Case 2 includes all flares in our sample that occurred during the same period (1969–70) and, in this case, the subdivision was made using our field directions. Case 3 includes all flares that occurred after this period, that is 1971–79, and case 4 includes our entire sample. The number of flares within each subdivision is indicated. The errors that appear against the average values of I are the standard errors of the means.

In case 1 the average value of I is significantly greater for flares with southward fields than for flares with northward fields. (A similar result is obtained using the CFI of Dodson-Prince *et al.*⁴.) This difference in average energies certainly would have given the flares with southward fields greater apparent geoefficiency and may partly explain the results of Pudovkin *et al.*

Table 2 Construction of flare energy index

Flare characteristic	Numerical weight	
H α Importance,	2	2
	3	3
H α Intensity,	Faint	0
	Normal	1
	Bright	2
X-ray class,	No event	0
	C	1
	M	2
	X	4
Radio type II,	No event	0
	Event	2
Radio type IV,	No event	0
	$P < 100^*$	2
	$P \geq 100^*$	4

* P is the product of the intensity (1, 2 or 3) and the duration (in minutes) of the Type IV burst as listed in the radio spectral reports.

*et al.*¹. This also has been suggested by Dodson *et al.*¹⁰ who went further to argue, on the basis of the sample of flares used by Pudovkin *et al.*¹, that large solar flares are significantly more likely to occur over southward field regions. We suggest that this apparent bias may be due to selection effects as this sample did not include all flares of importance 2 or greater during 1967–70. Our results show no significant bias of this kind within the complete sample of flares (from the group reports¹¹) which occurred during the partially overlapping period 1969–70 (case 2). The same conclusion can be drawn from cases 3 and 4 which show that the number and the average energy of flares with southward fields do not differ significantly from the number and the average energy of flares with northward fields. These results hold even for the very largest flares. Of the flares in our sample for which $I \geq 12$, 15 had southward fields and 11 had northward fields. Of the flares for which $I \geq 8$, 34 had southward fields and 32 had northward fields. Thus our results do not support those of Dodson *et al.*¹⁰. Furthermore, even if flares with southward fields were more energetic than flares with northward fields during the period 1967–70 it is doubtful whether the magnitude of the bias (case 1) is sufficient to explain the results of Pudovkin *et al.*¹. This is clear from Fig. 1 which shows only a small difference in $\bar{A}_p$ for flares with average indices of 5.1 and 9.3.

Next, we considered the geoefficiencies of flares of similar energies but with different field directions. Figure 2 shows the

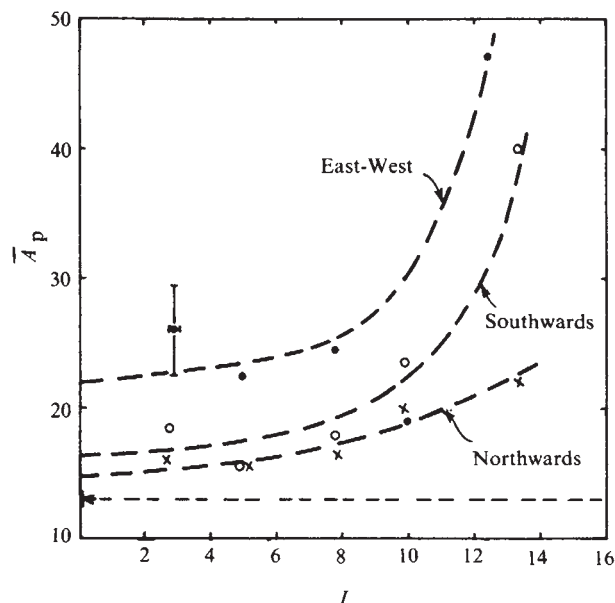


Fig. 2 Same as Fig. 1 except the data (from our entire sample) have been subdivided according to the inferred magnetic field directions at the sites of the flares. Results for flares with northward ($\times$), southward ($\circ$) and east-west ($\bullet$) fields are shown. The magnitude of typical r.m.s. errors of the means is indicated.

dependence of $\bar{A}_p$ on I when the inferred magnetic field direction at the flare site is taken into account. Three curves are presented, showing $\bar{A}_p$ plotted against I for flares with northward (N, NE, NW), southward (S, SE, SW) and east-west (E, W) magnetic fields. The scatter in the data points is larger than in Fig. 1 due to the smaller sample sizes involved. We have attempted to fit curves of the form shown in Fig. 1 to the data for each field direction. For flares with northward and southward fields, curves of this form fit the data points quite well. For flares with east-west fields, a curve of this form does not fit well but is reasonably compatible with the data points shown (note the data points at $I \approx 3$ and $I \approx 10$).

Several features of Fig. 2 are worth noting:

- (1) When $I \leq 10$, the magnitude of $\bar{A}_p$ for flares with southward fields only slightly exceeds the magnitude of $\bar{A}_p$ produced by flares, of similar energy, with northward fields.
- (2) When $I \geq 10$, the magnitude of $\bar{A}_p$ is significantly greater for flares with southward fields than for flares, of similar energy, with northward fields.
- (3) Flares with eastward or westward fields tend to produce higher values of $\bar{A}_p$ than flares, of similar energy, with either northward or southward fields.

Hence, the geoefficiency of solar flares appears to depend on the direction of the magnetic fields at the sites of those flares. However, in contrast to the results of Pudovkin *et al.*¹, flares for which the inferred field direction is southward do not

Table 3 Dependence of average flare energy on field direction

Case	Field direction					
	Southward (S, SE, SW)		East-west		Northward (N, NE, NW)	
	No.	Average I	No.	Average I	No.	Average I
1. Pudovkin <i>et al.</i> 1969–70	12	9.3 $\pm$ 1.4	12	6.2 $\pm$ 1.0	21	5.1 $\pm$ 0.5
2. This study, 1969–70	18	6.7 $\pm$ 0.9	19	6.2 $\pm$ 0.7	36	5.5 $\pm$ 0.5
3. This study, 1971–79	50	8.0 $\pm$ 0.5	31	6.5 $\pm$ 0.5	45	7.6 $\pm$ 0.5
4. This study, 1969–79	68	7.7 $\pm$ 0.5	50	6.4 $\pm$ 0.4	81	6.7 $\pm$ 0.4

have the highest geoefficiency. Clearly the relationship between field direction and geoefficiency needs reconsideration.

Pudovkin *et al.*¹⁻³ interpreted their results in terms of the well known association¹⁵⁻¹⁷ between geomagnetic disturbances and southward field in the solar wind. They argued that the orientation of the field within the flare site was preserved in the flare stream, thus accounting for the greater geoefficiency of flares for which the inferred field was southwards. However, the flare site is not confined to a narrow height range near the photosphere but extends upwards through the chromosphere, transition region and base of the corona¹³. The field direction may vary substantially with height within the flare. In the case of strongly sheared fields, the difference between the true magnetic field direction in the chromosphere, for instance, and the inferred direction in the underlying photosphere may be as great as $\pm 90^\circ$ (ref. 13). Thus even if the field direction within the flare is preserved in the flare stream it may not resemble

the direction inferred from the photospheric intensity contours. However, a systematic relationship may exist between the direction of the field within the flare and the orientation of the underlying photospheric field. If so it may help to explain the results of Fig. 2.

We have found no evidence to support the suggestion¹⁰ that major flares are significantly more likely to occur over regions of southward magnetic field. Therefore, we feel that it is not possible to interpret the results of Pudovkin *et al.*¹ in this way. Our results (Fig. 2) indicate that the inferred direction of the magnetic field beneath the flare controls, to some extent, the geomagnetic effects of solar flares, particularly outstanding flares. However, the relationship between the inferred field direction and the geoefficiency is not as distinct as stated by Pudovkin *et al.*¹ and needs further investigation. We suggest that the true magnetic field direction within the flare site may be more relevant to the task of predicting geomagnetic effects of solar flares.

Received 31 December 1981; accepted 2 July 1982.

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Direct imaging of travelling Rayleigh waves by stroboscopic X-ray topography

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We report here the first successful experiments which exploit the time structure and single bunch mode of operation of the Daresbury Synchrotron Radiation Source (SRS). Rayleigh waves travelling on the surface of a piezoelectric crystal have been imaged by stroboscopic X-ray topography, in which the generation of the waves is synchronized with X rays emitted by the orbiting electrons in the storage ring. Interactions between the Rayleigh waves and microscopic crystalline defects have been observed and provide new insights into the factors affecting surface acoustic wave (SAW) device operation. It has been demonstrated that this novel technique has considerable potential for the study of periodic phenomena in crystals.

Piezoelectric SAW devices¹ are of great technological importance for high-frequency signal processing and filtering applications. Although the presence of crystallographic defects can have deleterious effects on the characteristics of these devices, there has been almost no study of the interactions between the Rayleigh waves and the defects concerned. Techniques such as electrostatic² and optical probes³ can be used to observe the waves, but these give no information concerning lattice strains or bulk crystal defects. Stroboscopic scanning electron microscopy has been applied to the study of piezoelectric Rayleigh waves⁴ but only surface defects and charges are imaged. X-ray topography⁵, a diffraction technique by which a two-dimensional map of the lattice strains in a crystal can be obtained, has been applied⁶ to reveal the average strain associated with a travelling Rayleigh wave but standard X-ray illumi-

nation gives no information about the periodic strains in the wave. Such strains can be revealed by generating standing waves although this gives information of limited relevance as most delay lines and filters operate by propagating travelling waves.

The unique time structure of X rays emitted from a storage ring can be exploited for stroboscopic X-ray topography in which the generation of the waves is synchronized with the illuminating pulses of radiation. Recent, independent experiments⁷ at Hamburg have demonstrated the feasibility of such a technique from studies of standing bulk waves in quartz.

Our experiments were performed with the Daresbury SRS running in the single bunch mode at 2 GeV 1.8 mA with a lifetime in excess of 30 h; 97% of the synchrotron radiation was concentrated in a single pulse of width ~ 200 ps. A stroboscopic timing pulse was obtained from the SRS ring monitors. This 3.122 MHz square wave was frequency quadrupled, filtered and amplified to provide a 37.46 MHz, 15 V p/p sine wave to drive a 38 MHz LiNbO₃ television IF filter. White radiation X-ray topographs of the Y cut Z propagating LiNbO₃ crystal were taken using the multipurpose diffractometer at the

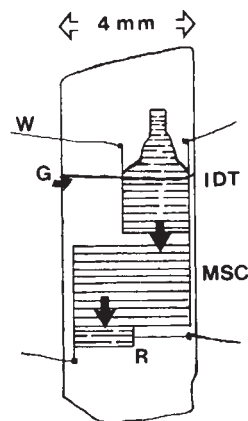


Fig. 1 Electrode configuration on the surface of the SAW device. The wave is generated in the interdigital transducer IDT and is transferred across the multistrip coupler MSC. It is detected at the receiving transducer R. W are the bonding wires, G marks the grain boundary visible in Figs 2 and 3.

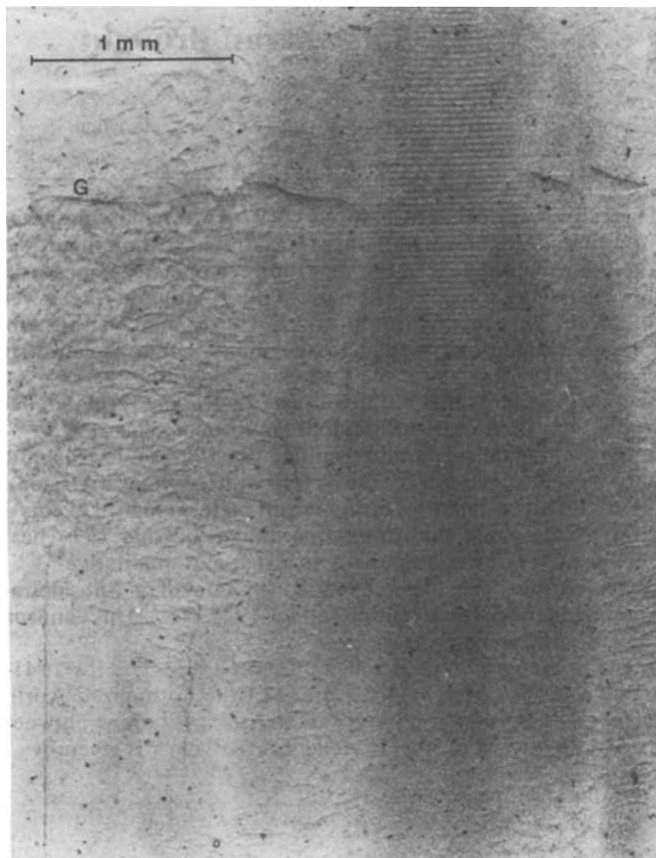


Fig. 2 X-ray topograph taken of the Rayleigh waves without synchronization to the synchrotron radiation emission. The diffuse streaks correspond to the SAW strains, the fine curved lines to dislocations. The black line G is the sub-grain boundary indicated in Fig. 1. (The measured spacing of the standing waves ($33\ \mu\text{m}$) is reduced from the half wavelength value of $46.5\ \mu\text{m}$ by a factor of $\cos 45^\circ$ due to geometrical image distortion.) 0330 reflection, 45° Bragg angle. Specimen to plate distance, 35 mm.

SRS Topography Station⁸. LiNbO_3 has a space group $R3c$ and lattice parameters $a = 5.147\ \text{\AA}$ and $c = 13.856\ \text{\AA}$. In the hexagonal indices Y corresponds to $[01\bar{1}0]$ and Z to $[0001]$. For these experiments the $[01\bar{1}0]$ direction (normal to the crystal surface) was set at 45° to the incident X-ray beam. All topographs presented were taken in the Bragg geometry on Ilford L4 Nuclear Emulsion with exposure time of $\sim 1\ \text{h}$. The many topographs simultaneously recorded correspond to a large spot size, high spatial resolution back reflection Laue pattern. A Rayleigh wave was excited by an untuned interdigital transducer visible in the upper region of Fig. 1. The electric fields associated with it are transmitted laterally along a multi-strip coupler deposited on the surface. Thus a new acoustic wave is generated which propagates, parallel to the original wave, to the receiving transducer.

Figure 2 shows an X-ray topograph taken with the Rayleigh wave propagating but without the oscillator synchronized with the electron orbit frequency. Standing waves excited within the transducer are visible in the upper region and an increase in intensity due to the mean strain associated with the travelling wave emanating from it can be observed. Figure 3a shows part of a similar topograph taken with the driving signal phase locked to the synchrotron radiation emission as described above. A periodic array of dark and light bands is visible, an image of the strains in the crystal due to the travelling Rayleigh wave at an instant in the cycle. At 37.46 MHz, the wavelength of the acoustic waves is $93\ \mu\text{m}$ in LiNbO_3 and it is seen that, when the appropriate geometrical correction has been made for image distortion, the contrast pattern on the topographs corresponds to a period of a half wavelength. This occurs because strains

of either sense give rise to an increase in scattered intensity in white radiation topographs taken in the present conditions. Light areas, of intensity equal to the background, correspond to regions under very small strain at that instant of the propagating cycle. Detailed study of the visibility of the SAW in the many reflections recorded simultaneously on the photographic plate will enable the strain components to be determined.

Insertion of a time delay into the driving signal line enables the strains associated with different instants in the cycle to be examined. The result of inserting a 6 ns delay is shown in Fig. 3b. Light and dark areas have now interchanged, as is expected by moving back approximately a quarter of a cycle. Comparison of Fig. 3a with 3b reveals that the wavefronts are not always continuous throughout the cycle. At points X and Y, two 'dislocations' in the wavefronts are visible in Fig. 3a which are not present a quarter of a cycle later. Clearly some form of interaction is occurring periodically, a feature which cannot be detected other than by stroboscopic methods. Careful examination of the topographs reveals a phase shift of $\pi/2$ in the

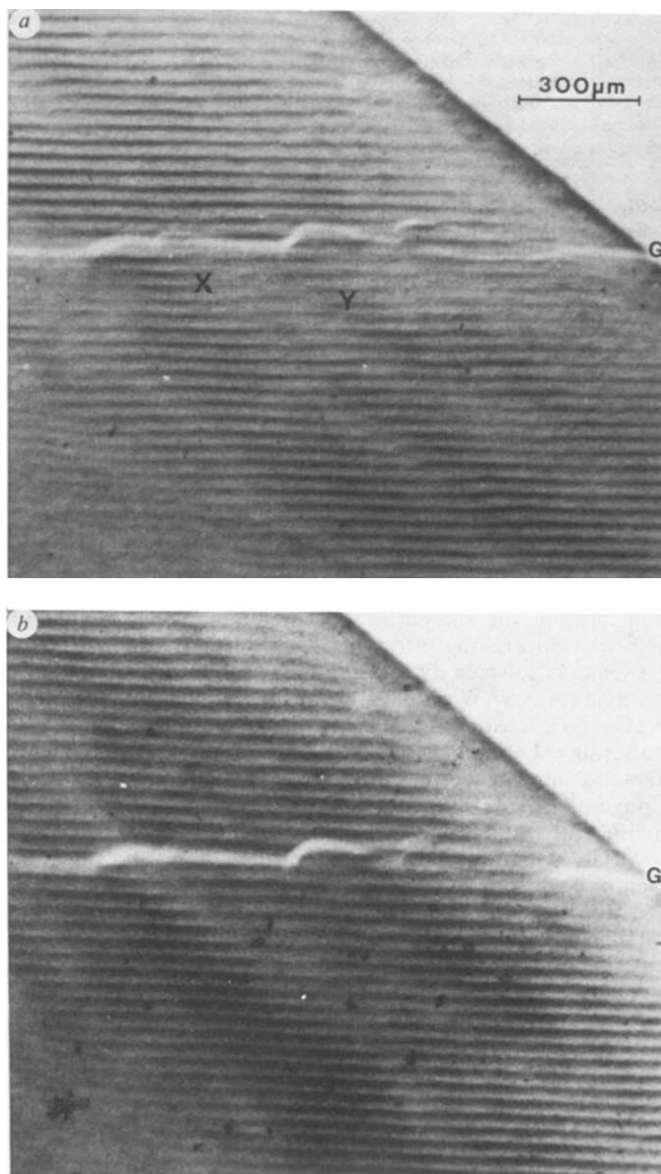


Fig. 3 Stroboscopic X-ray topographs of travelling Rayleigh waves taken with the driving signal phase locked to the synchrotron radiation emission: a, no time delay; b, additional 6 ns time delay. The broad white line G corresponds again to the sub-grain boundary marked in Figs 1 and 2. $\bar{1}321$ reflection predominant, 33° Bragg angle. Specimen to plate distance 20 mm. Projection of the diffraction vector is parallel to the crystal edge.

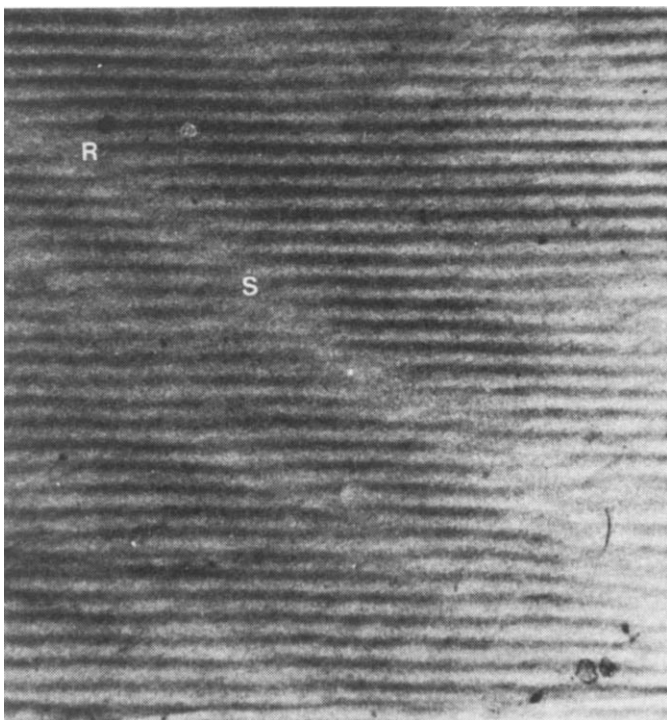


Fig. 4 Interaction of Rayleigh waves with lineal defects in the lower region of the multistrip coupler MSC. Note a phase shift of almost $\pi/2$ across the line RS. Same diffraction conditions as in Fig. 3. In both Figs 3 and 4, the wave propagates down the page.

acoustic wave along the multistrip coupler as predicted by Maerfeld⁹. In Fig. 4 we see a very strong interaction, present throughout the cycle, between a linear defect in the lower region of the multistrip coupler and the Rayleigh waves. The waves are phase shifted by about $\pi/2$ on crossing this defect, introducing another 'dislocation' into the images of the wavefronts. At present the nature of the defect has not been established.

Our experiments have established a totally new diagnostic tool for SAW device technology. Future studies will concentrate on Rayleigh wave propagation from transducers of varying configuration, the analysis of unwanted bulk modes excited by such transducers, the interactions with crystal lattice defects, the relation between diffracted intensity and wave strains, and the changes in SAW propagation as the frequency is increased. We have demonstrated that high-frequency stroboscopic X-ray scattering experiments are remarkably easy to implement with standard film recording techniques. In addition to X-ray topography, several diffraction experiments can now be envisaged to study rapid periodic phenomena in such diverse materials as thin films, ceramics, powders, fibres, polymers or even biological materials. The unique, extremely stable, time structure of the SRS running in single bunch mode has thus far not been the focus of other proposals for X-ray scattering experiments. Our present success and the availability to users of single bunch operation will hopefully stimulate many new experiments using these 200 ps width light pulses.

Financial support from SERC is acknowledged. We thank Dr P. J. Duke and Mr D. Warne for assistance in setting up these experiments.

Received 17 June; accepted 21 July 1982.

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Persistence of Subsaharan drought

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The Subsaharan drought of the early 1970s attracted widespread attention. Particularly prominent was the suggestion that it was not likely to disappear in the near future, and could even persist into the next century¹⁻³. (The basis of this standpoint is reviewed in ref. 4.) In contrast, the post-1974 discussion of the Subsaharan climate has tended to the opinion that the drought ended by 1974 or 1975⁴⁻¹¹. That this may not be the case has been neglected. It was not investigated during several detailed studies of West African rainfall published since 1975¹²⁻¹⁶, because the data sets used terminated in 1974 or 1975, and is only weakly suggested by the World Meteorological Organization's annual summaries of significant meteorological events¹⁷. Strong claims that the drought continued beyond 1974 have been few, very brief, regionally focused, and qualitative¹⁸⁻²⁰. I therefore now provide a 1975-81 update of a Subsaharan rainfall index previously published for 1941-74²¹. This confirms the drought's persistence.

The Subsaharan rainfall index presented (Fig. 1) is the 1941-81 time series of the yearly average of the normalized April-October departures for 14-20 West African stations between 11 and 18°N west of 9°E. Its value for the year j is given by

$$\chi_j = \frac{1}{N_j} \sum_{i=1}^{N_j} \frac{r_{ij} - \bar{r}_i}{\sigma_i}$$

where r_{ij} is that year's April-October rainfall total at station i , $\bar{r}_i$ and σ_i are respectively the mean and standard deviation of station i 's April-October rainfall for 1941-74, and N_j is the number of stations with complete records in year j . This index has never been evaluated before 1941 (ref. 21, Fig. 1; present Fig. 1) because only 6 of the 20 possible stations were operative then, one of which commenced in 1940. The remaining stations were established in 1941 (8), 1947 (1), 1950 (4) and 1951 (1). All 20 are located in Fig. 2 of ref. 21, which shows their adequate spatial distribution. These stations, which possess the most reliable and readily accessible records for the region, are those that appear in standard data publications²²⁻²⁶. Individual monthly station rainfall totals were obtained from these sources and various national and regional meteorological services. The April-October interval used includes both the July-September rainy season characteristic of the entire region, which on the average provides 80% of the annual rainfall²⁷, and the adjacent months that collectively contribute a further 17% (ref. 27). The continued adoption in this 1975-81 index update of the 1941-74 base period for $\bar{r}_i$ and σ_i utilized in the original publication of the index for that period²¹, maintained the continuity of the time series in Fig. 1. For the proper use of a regional index of the present type, the rainfall departures must be statistically coherent throughout the region concerned. This has recently been strongly confirmed for Subsaharan West Africa^{13,15,16,18}. The statistical advantages of the particular index used here have been summarized by Kraus¹³.

The depiction of the index in Fig. 1 is complemented by the documentation in Table 1 of the consistency of the individual station departures for the years since the start of the aforementioned drought (1968), and also the dry years of the 1940s. These displays clearly suggest that the drought has not ended, despite the pronouncements to the contrary mentioned above⁴⁻¹¹. Although rainfall increased progressively from the especially severe 1972 drought year until 1975, this recovery did not reach even the 1941-74 mean value. Furthermore, it was immediately followed by a counterbalancing decrease in 1976-77. As a result, the 1977 rainfall was as deficient as that of 1972, and much more so that the other well-publicized severe drought years of the late 1960s and early 1970s (that is, 1968,

1970, 1971, 1973). While Subsaharan rainfall was clearly much greater in 1978 than a year earlier, this recovery also failed to reach the average value for 1941–74. Moreover the three most recent years have been characterized by a further progressive rainfall decline. This was sufficient to render 1979 and 1980 as dry as 1968 and 1970 and almost as rainfall deficient as 1971 and 1973, and to leave 1981 drier than all post-1967 years except for the very severe drought years of 1972 and 1977. The occurrence of the 1981 drought received little mention in the *Climatic Impact Assessment, Foreign Countries, 1981 Annual Summary* published by the United States' Center for Environmental Assessment Services (CEAS)²⁸; this organization now routinely monitors the globe's 'major anomalous meteorological events' and their 'adverse impacts'²⁸. Two of the seven 1975–81 years (1977 and 1981) also appear to have received substantially less rain than the better known drought years of the 1940s (Fig. 1). In addition, 1979 and 1980 were probably at least as dry as any year in the 1940s. Finally, it is thus clear that rainfall above the 1941–74 average has not returned to Subsaharan West Africa as a whole in the years since drought and famine made this region such a focus of attention in the early 1970s. This has extended the period since the last era of abundant rainfall (1950–58) to almost 25 yr.

The foregoing results have interesting implications. First, the suggestion that the Subsaharan drought was not likely to disappear in the near future^{1–3} has so far not been disproved. This fact, of course, does not constitute an endorsement of the basis of that prediction. Second, there is as yet no support for Faure and Gac's¹⁹ recent extrapolation-based projection of a gradual recovery from the present drought to more average rainfall during 1975–85. Third, the fact that the present drought has extended from 1968 to at least 1981 (14 yr), which is markedly longer than the three other distinct climatic epochs evident in Fig. 1 (1941–49, 1950–58, 1959–67; all of 9 yr duration), supports Kraus'¹³ findings that the Subsaharan rainfall record contains "unexpectedly long runs of wet or dry years" and that its droughts "tend to be rather persistent". This is notwithstanding Katz's²⁹ claim that the anomaly values possess "only a small degree of year-to-year persistence". Next, it should be emphasized that the occurrence of severe drought in 1977 and 1981 makes these years ideal candidates for case study investigations of the coincident (and presumably causal) tropical circulation anomalies in the manner already performed for 1972 (see ref. 10) and 1968 (see ref. 30). Finally, Glantz's³¹ 1977 belief that "with a return to above-mean rainfall levels, interest in dealing with (the) chronic (Sahelian) problems (of inadequate management practices and institutional arrangements revealed by the drought) will dissipate" now takes on added significance. The lack of publicity given to the continuation of the drought between 1975 and 1981 could well mean that such interest has

Table 1 April–October rainfall departure statistics for Subsaharan West Africa for 1968–81 and the drought years of 1941–49

Year	Fraction of stations with rainfall departure $\geq -1.0\sigma$ from 1941–74 mean	Fraction of stations with rainfall departure $\geq -0.6\sigma$ from 1941–74 mean	Average of all stations departures (σ)
1968	7/20	14/20	-0.78
1969	2/20	4/20	+0.06
1970	8/20	13/20	-0.72
1971	9/20	15/20	-0.88
1972	17/20	19/20	-1.39
1973	13/18	16/18	-0.91
1974	7/16	10/16	-0.60
1975	3/20	8/20	-0.27
1976	7/20	10/20	-0.62
1977	13/18	15/18	-1.42
1978	5/20	10/20	-0.42
1979	8/20	14/20	-0.70
1980	9/20	13/20	-0.80
1981	9/20	15/20	-1.00
1941	4/14	7/14	-0.67
1942	7/14	8/14	-0.63
1947	3/15	7/15	-0.46
1949	5/14	7/14	-0.58

evaporated even without a rainfall recovery. This may have been facilitated by the region's drought-induced population (human and animal) decline of the early 1970s reducing the socio-economic impact of the more recent deficient rainy seasons. The other alternatives—that the local infrastructure has dramatically improved and/or western aid has been suddenly beneficial—are more unlikely.^{9,31,32}

In view of the foregoing results and their implications, it is planned to update the present Subsaharan rainfall index towards the end of every calendar year.

Some of this material was first presented at the August 1981 Oxford Symposium on Variations in the Global Water Budget, with the aid of an award from the Legacies Fund of the Royal Meteorological Society and the encouragement of Stanley A. Changnon Jr. Grateful acknowledgment is also made of the data provided by West African regional and national meteorological services, and the assistance of P. Stone, W. Wendland, K. Hendrie and E. Kraus.

Received 30 April; accepted 29 June 1982.

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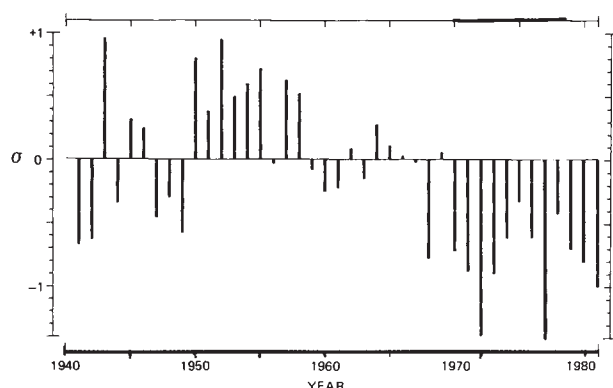


Fig. 1 1941–81 time series of the yearly average of the normalized rainfall departures (σ) for 14–20 Subsaharan stations west of 9°E. Kraus¹³ and Faure and Gac¹⁹ suggest that the largely dry epoch at the start of the time series (1941–49) commenced no more than a year or two earlier. The 1969 value of +0.06 is a correction of the +0.11 value published earlier²¹.

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Late Palaeozoic Gondwana glaciation in Oman

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Late Carboniferous–early Permian sediments belonging to the Haushi Group in Oman have been considered to be partly of glacial origin^{1–3}. However, conclusive evidence of a continental glaciation has been lacking, and the extension of the late Palaeozoic Gondwana glaciation into the Arabian Peninsula has not been generally accepted⁴. During recent visits to outcrops of the Haushi Group in the Al Khlatra area of the Huqf Massif (Fig. 1), we have found good exposures of striated pavements of Precambrian dolomite, which are directly overlain by diamictites of the Haushi Group (Fig. 2). This occurrence forms the strongest evidence so far for the presence of a continental glaciation in Oman during the late Palaeozoic.

Previous references to the occurrence of late Palaeozoic glacial sediments on the Arabian Peninsula have been summarized elsewhere^{5,6}. Roland⁵ describes the occurrence of striated and faceted pebbles and boulders of granites, gneisses and amphibolites in the Akbra shales of North Yemen and interprets them as dropstones from icebergs in a glaciomarine environment. Although no dating is mentioned, he considers the Akbra shales to be of late Palaeozoic age. McClure⁶ presents new evidence for a Stephanian to Sakmarian (late Carboniferous–early Permian) age of boulder beds in the Wajid Sandstone Formation at Khashm Khatmah and Jabal Umm Ghiran in Saudi Arabia (Fig. 1). He suggests that these boulder beds represent fluvially-reworked glacial deposits, but states, referring to the Arabian Peninsula as a whole: "Unfortunately, definitive indication of glaciation such as striated pavement and tillite is lacking".

In the context of a broader investigation into the sedimentology of the Haushi Group, exposures were studied in the outcrop area bordering the Huqf Massif (Fig. 1). Here the Haushi Group rests with an erosional unconformity on Precam-

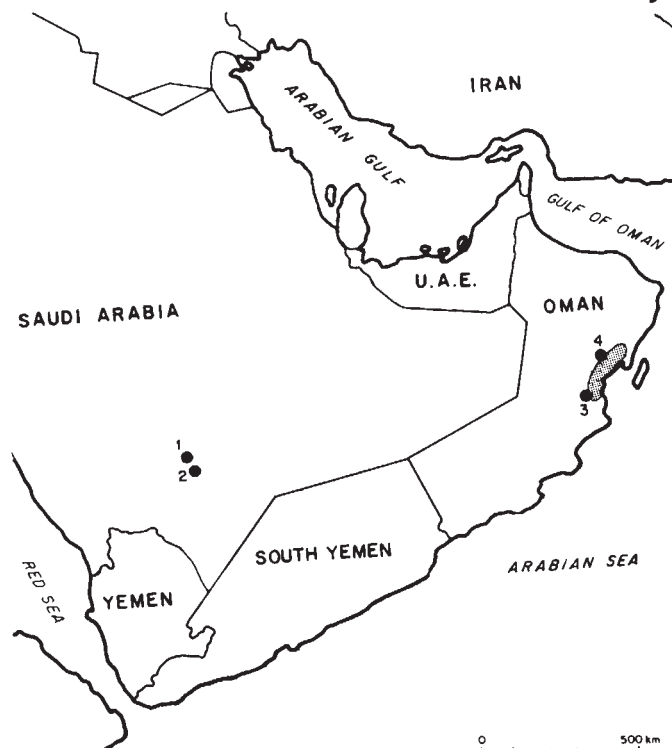


Fig. 1 Location map: 1, Jabal Umm Ghiran; 2, Khashm Khatmah; 3, Al Khlatra; 4, Haushi. (The Huqf Massif is shaded.)

brian formations of the Huqf Group. In the Al Khlatra area this unconformity is well exposed in three different locations, forming a triangle of some 2 km². On the resistant surface of stromatolitic dolomites of the Huqf Group, parallel sets of striations and grooves (up to 35 cm wide and 10 cm deep) can be traced across the exposures, which are up to 30 m wide. Individual striae are up to 15 m long (Fig. 2). At this locality, 18 striae were measured (vector mean $45^\circ \pm 3^\circ$, vector magnitude 99.8%). In the other two localities, striae again have consistent mean azimuths of 45° although in one exposure a second set with an azimuth of 30° was observed. Direction of glacier movement could not be established from the striated pavements, but the consistent northeasterly foreset directions in fluvial sandstones and conglomerates which erosionally overlie the diamictite, suggest, in a simple palaeogeographical view, ice movement towards the north-east. The glacial sedimentary sequence appears to be onlapping onto the Huqf Massif, sediment transport being towards or along its margins. In two of the exposures a diamictite unit, with an average thickness of 4 m, rests directly on the striated dolomite (Fig. 2) and is therefore interpreted as a basal till. This diamictite is well exposed and correlatable over a distance of at least 1.4 km. Within a grey, fine-grained matrix it contains both igneous and sedimentary clasts. The sedimentary material consists of angular, centimetre-sized pieces of argillite and black chert (which may have been derived from the Precambrian Shuram Formation, outcropping 10 km south of Al Khlatra) and fragments of dolomite probably derived from the local bedrock. The better-rounded pebbles and boulders of granite and other igneous rocks have not been related to any *in situ* occurrence in Oman. According to our present knowledge, no granites are present in surface or subsurface within a radius of 250 km of Al Khlatra and hence the granite clasts are indicative of long-distance transport.

Further evidence for glacial deposition is found in several good exposures in the same area: sequences of rhythmically bedded or laminated fine sandstones and mudstones contain occasional outsize clasts (up to 240 cm), interpreted as dropstones from melting ice, floating on a body of standing water (Fig. 3a).

Table 1 Criteria for the identification of glacial deposits and their applicability in Oman

	Al Khlatra	Subsurface
Abraded, striated bedrock surfaces	A	—
Stone-rich beds (diamictites)	A	A
Depositional fossil land forms	—	—
Dropstones	OP	A
Finely-graded stratification ('varves')	PP	A
Great thickness and extent of diamictites	—	PP
Association tillites/resedimented deposits	PP	OP
Variable clast lithology	A	A
Stones with wide range of shapes	A	A
Striated and faceted stones	OP	—
Clay-sized particles ('rock flour')	A	A
Fragile stones	OP	PP
Typical quartz grain textures	?	PP

A, abundant or well-defined; OP, occasionally present; PP, probably present; ? doubtful; —, not observed. Criteria after ref. 11.

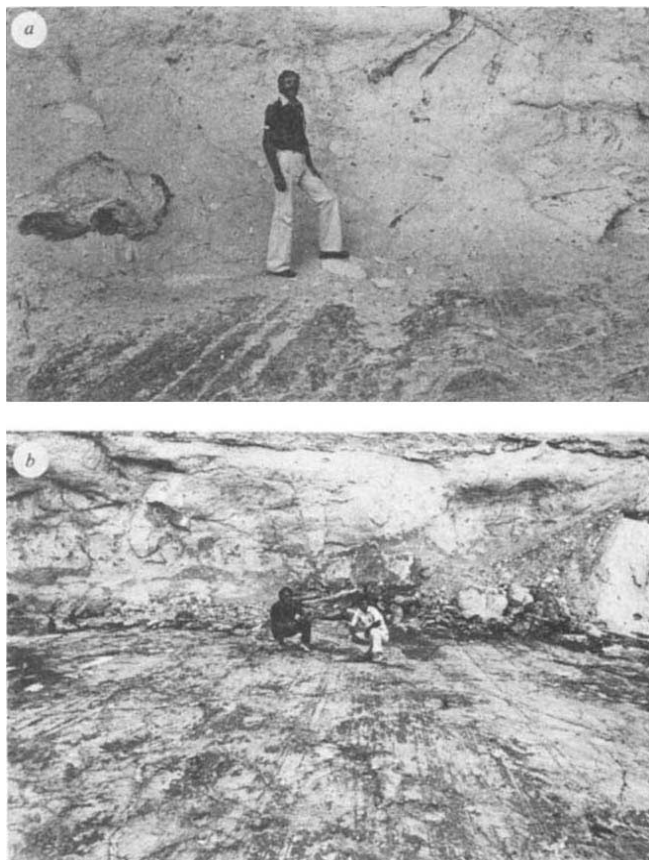


Fig. 2 *a*, Parallel grooves on pavement of Precambrian dolomite, overlain by a 4 m-thick diamictite (Al Khlat area). Photograph taken towards NE. *b*, Striated pavement in same exposure. Photograph taken towards SW.

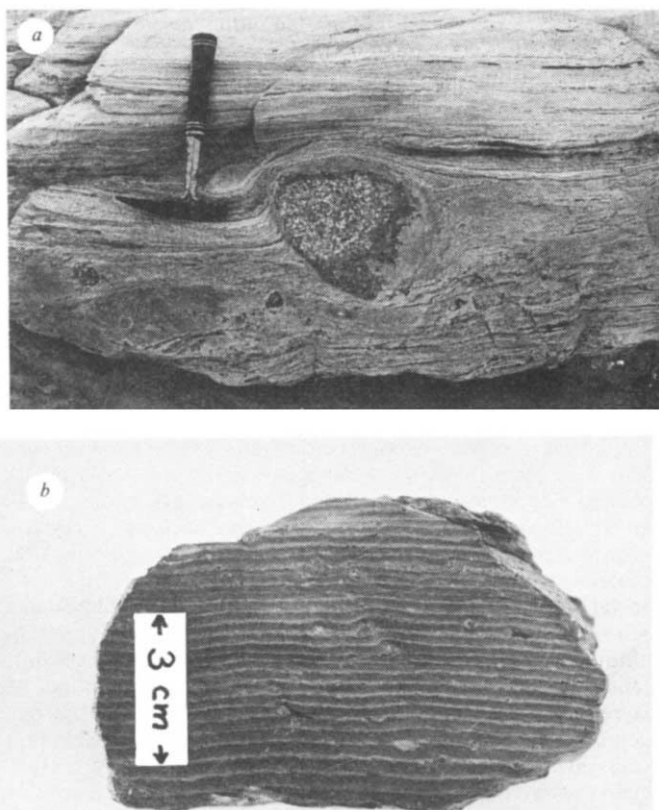


Fig. 3 *a*, Granite pebble and other dropstones in rhythmically bedded siltstones (Al Khlat area). *b*, Varved shale with dropstones from a cored well (South Oman).

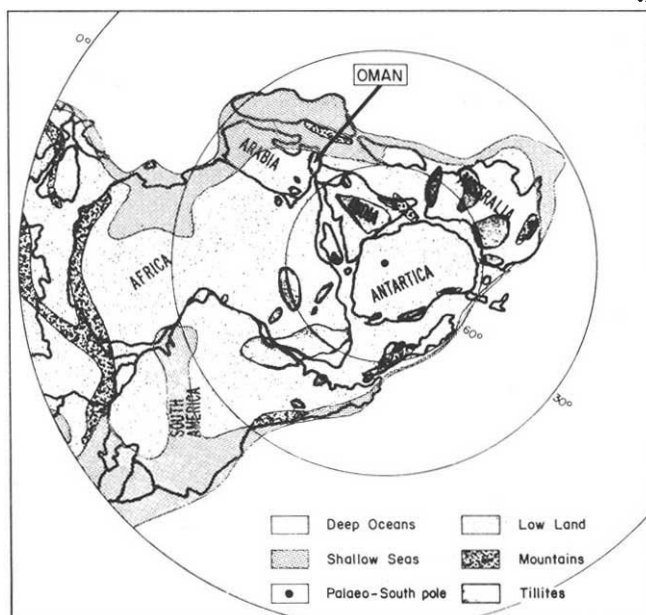


Fig. 4 Palaeogeography of the Southern Hemisphere during the late Carboniferous (after ref. 13).

Interbedded with the dropstone-laminites, discontinuous bodies of diamictite and poorly-sorted sandy conglomerates occur, some of which contain striated dolomite boulders. These units are interpreted as sub-aqueous mud flows and debris flows. Although the general appearance of these diamictites is similar to that of the basal diamictite, stratigraphical relationships favour an interpretation as subaqueously redeposited glacial debris.

Without the presence of the sedimentary units described above, which show strong evidence of a glacial environment, it would be difficult to place the bulk of the Haushi Group sequences as exposed in the Al Khlat area in a glacial setting, as it consists mainly of cross-bedded sandstones and conglomerates of fluvial or fluvio-deltaic origin.

The age of the Haushi Group in the outcrop area has been well established by palynology. The diamictites and associated claystones from the Al Khlat exposures have yielded well-preserved palynomorph assemblages. The microflora consists largely of *Cristatisporites* sp., *Parasaccites* sp., *Potoniesporites* sp., *Punctatisporites* sp., *Vallatisporites* sp. and *Verrucosporites* sp. Less common constituents include bisaccate non-striate and bisaccate striate pollen and a distinctive species, *Ahrensia* sp. *cristatus* Playford and Powis. The assemblages resemble those illustrated by Kemp⁷ from the Permo-Carboniferous glacial sequences of Pakistan and Brazil, and display the general characteristics of those which are assigned a latest Carboniferous age by Balme⁸. A considerable amount of reworking of palynoflora might be expected in a glacial environment; however, the presence of multistriate pollen precludes a dating older than latest Carboniferous. A bioclastic limestone interval conformably overlying the sequence containing the boulder beds in the Haushi area (Fig. 1) has been assigned an age no younger than earliest Artinskian, probably Sakmarian (early Permian)⁹, thus confirming the late Carboniferous to early Permian age of the diamictite sequence in Al Khlat.

The new evidence presented here supports the concept of a glacial to periglacial origin for the lower part of the Haushi Group in Oman as accepted and used¹⁰ by Shell and PD Oman geologists since 1976, when the occurrence of varved shales with dropstones was reported in cored wells (Fig. 3b).

Table 1 summarizes the features which are generally considered to be indicative of a glaciation, showing which of these are found in the Haushi Group of Oman. The presence of the striated bedrock and the occurrence of dropstones are considered the most diagnostic characteristics of glacial activity¹¹.

The significance of this discovery in relation to the reconstruction of Gondwanaland during the late Palaeozoic still has to be assessed. Interpretations of the palaeolatitudes of Oman during this period vary considerably. Some reconstructions¹² show a low latitude ($<40^\circ$) for Oman during this period, but a 1979 reconstruction by Scotese *et al.*¹³ shows Oman at about 50° during the late Carboniferous, which is a similar palaeolatitude to that assumed for the well-documented glacial deposits in South America, South Africa and Australia (Fig. 4). Therefore, the Permo-Carboniferous glaciation in Oman could serve as support for this reconstruction of Gondwana.

Finally, we have recently learned from Thiele (personal communication) that further evidence for a late Palaeozoic glaciation has been found in North Yemen in the form of striated bedrock (granite and gabbro), overlain by tillite belonging to the Akbra Formation¹⁴. Preliminary dating of this formation by palynology suggests an age younger than Namurian and probably Permian.

Internal company reports, using data of the more than 300 subsurface well penetrations of the Haushi Group have greatly contributed to our knowledge of these glacial deposits and stimulated further studies leading to this article. Special acknowledgement is made to the work of F. J. Winkler of Shell Expro, London. We thank Shell Research B. V., The Hague, and Petroleum Development Oman LLC for their permission to publish this paper, which appears with the approval of H.E. The Minister of Petroleum and Minerals of the Sultanate of Oman.

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Volatile C₁–C₈ organic compounds in macroalgae

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Small amounts of C₁–C₈ volatile organic compounds were found in macroalgae in concentrations high enough (generally in the range of 1–400 times those in sediments) to suggest that the seaweeds are a source for some of the volatile organic compounds identified in recent marine sediments. The types of compounds identified^{1–4} include alkanes, alkenes, aldehydes, ketones, furans, and sulphides. Both the light hydrocarbons and the volatile functionalized organic compounds are believed to originate from living organisms and from both biological and chemical low-temperature reactions in the sediments. Understanding their mechanism of formation may help in deciphering the thermal and biological history of the sediments. Traces of some of these compounds can be produced from bacterial degradation of naturally occurring terpenoids⁵. We consider here their occurrence in algae.

Four species of seaweeds, *Ulva lactuca*, *Ascophyllum nodosum*, *Gracilaria tikvahiae* and *Hypnea musciformis* were

grown in 1,700-l aerated tanks using circulating seawater. The macroalgae (grown and maintained by J. Ryther of Woods Hole Oceanographic Institute) were selected because of their high productivity and ease of culturing. All the species studied were shallow water species often found in tidal zones. *U. lactuca* is found from the tropics to Newfoundland, *A. nodosum* from New Jersey northward, *G. tikvahiae* is from the tropics and *H. musciformis* grows from the tropics to the south coast of Massachusetts.

Algae samples were analysed by a modified head space technique which has been used to investigate volatile organic compounds in sediments³. The plants were disaggregated in a stainless steel blending assembly equipped with Teflon gaskets and a screw cover with a sampling septum. Each sample (20–40 g wet weight) of algae was placed in the blender with distilled degassed water leaving a 118 ml gas headspace. The blender was then placed inside a glove bag which was aspirated to exclude air and filled with helium. The vessel was sealed while inside the bag, its contents were blended at high speed for ~10 min, and then heated in a 90 °C water bath for 30 min. Samples were removed through the gas chromatography (GC) septum using a syringe and analysed by GC and GC-MS⁶. Degassed distilled water and the tank water in which the algae were grown were analysed as blanks. None of the compounds reported was detected in the blanks. Compound identification was based on a comparison of GC and GC-MS data with those of commercially available standards.

Amounts of compounds present in various species of algae are shown in Table 1. Except for the sulphides, none of these compounds have previously been reported in algae. Most of the compounds found in macroalgae also occur in surface marine sediments, but usually at lower levels (~0.1–100 ng per g)^{2,3,6}. The alkenes are found most frequently in marine sediments with an oxic sediment–water interface^{2,6}. They generally decrease to trace levels (<0.1 ng per g) in the top metre of sediment.

All species of algae in Table 1 contained *n*-pentane in concentrations >10 ng per g. No other pentane isomers were detected. The discovery that recent marine sediments generally have a low isopentane to *n*-pentane ratio could be partly explained by algal contributions of *n*-pentane⁷.

The aldehydes, ketones, furans, and sulphides of Table 1 are frequently found in marine sediments—for example, on the Walvis Bay Shelf, the Peru Shelf, and the Gulf of Maine^{2,3,6}. Their levels are in the range 0.1–100 ng per g in reducing sediments. However, they also occur sporadically in smaller amounts (0.01–10 ng per g) in sediments with oxic sediment–water interface, such as the Gulf of Maine and parts of the Peru Shelf. Some of these compounds have also been detected in more deeply buried (>100 m subbottom) sediments where the geothermal gradient is low enough that the compounds have not been exposed to temperatures >20 °C, such as DSDP sites 438 and 436 in the Japan Trench⁴.

It was previously proposed that some of the volatile organic compounds in sediments may be derived from terpene precursors which occur in marine organisms³. This work supports this concept. Algae provide a rich and diverse range of these terpenes^{8,9}. Large areas of these plants can be seen in surface ocean waters in many parts of the world. Even though the occurrence of various species of macroalgae in oceanic and coastal waters is patchy, the sinking of these large plants and their fragments would provide a way of rapidly delivering small, volatile molecules to the sediments with minimal water column biodegradation. Little is known about the contribution of macroalgae to organic matter on the deep sea floor¹⁰. It has been estimated that even in deep water, large particle sinking occurs in a matter of days¹¹. The macroalgae plant structure would prevent loss of these very volatile compounds and protect the more labile functional groups, such as the aldehydes, from biodegradation at the sediment–water interface where intense microbiological activity occurs¹². Because biological activity in sediments decreases rapidly with depth¹² burial of plant

fragments to depths of even a few centimetres could provide some protection of these molecules from biodegradation.

Although much recent work has concentrated on identifying larger molecules in macroalgae¹³, the smaller, more volatile components shown in Table 1 have been largely ignored. Certain compounds seem to be restricted to particular species: methyl-1, 3-cyclopentadiene in *H. musciformis* and three compounds in *G. tikvahiae* tentatively identified as 2-ethyl-cyclobutanol, cycloheptene, and 3-cycloheptene-1-one, based on a search of the NIH 30,000 mass spectral library¹⁴. An unusual feature of the latter two compounds is their mass spectral base peak at $m/z = 54$. The 3-cycloheptene-1-one occurs in very large amounts in *G. tikvahiae*. It is either very volatile or labile as the amount measured in material which had been frozen for several months had decreased from 1 mg per g to 3,100 ng per g. In frozen samples a similar decrease in concentration over time did not occur for other compounds shown in Table 1. The literature on natural products from red algae, of which *G. tikvahiae* is a member, does not report any structures comparable to the cycloheptene, 3-cycloheptene-1-one, or the ethyl cyclobutanol, although cycloheptadiene derivatives have been reported in brown algae¹⁵. These volatile compounds would be lost in normal isolation procedures which generally involve extraction with organic solvents.

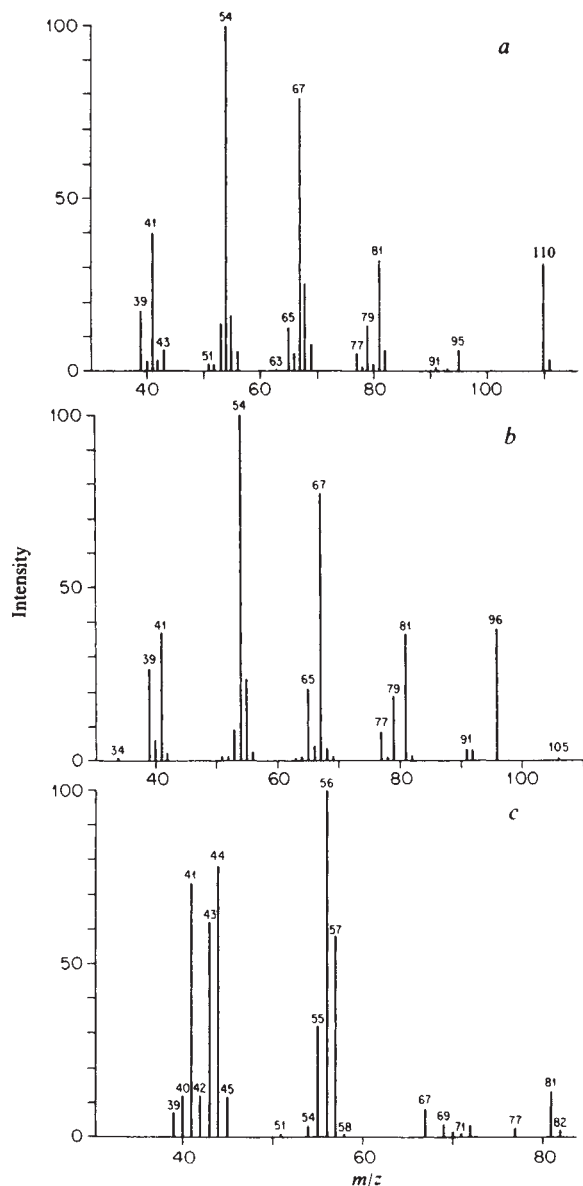


Fig. 1 Mass spectra of compounds from *Gracilaria tikvahiae*. Tentative compound identification: a, 3-cycloheptene-1-one; b, cycloheptene; and c, ethylcyclobutanol.

Table 1 Amounts of volatile organic compounds found in various species of macroalgae (units ng of compound per g dry weight of algae)

Compound	<i>Ulva lactuca</i>	<i>Hypnea musciformis</i>	<i>G. tikvahiae</i> (nutrient enriched)	<i>G. tikvahiae</i> (nutrient deprived)	<i>Asco-phylum nodosum</i>
1-Butene	0	>50	0	0	0
n-Butane	6	0	55	ND	0
1-Pentene	0	0	0	0	11
n-Pentane	10	16	171	Present	110
3-Methylpentane	0	22	0	0	0
1-Methyl-1, 3-cyclopentadiene	0	114	0	0	0
Benzene	20	20	0	0	0
Dimethylsulphide	>2,700†	>79,000	>2,000,000	0	>19,000‡
Dimethyldisulphide	0	>4	0	0	0
Propanal	0	480	0	0	0
2-Methylpropanal	190	310	0	0	0
Butanal	0	630	1,900	750	0
3-Methylbutanal	0	120	800	260	0
Pentanal	0	2,500	310	1,900	1,200
Hexanal	0	0	0	7,000	0
Acetone	0	0	0	0	>19,000‡
2-Butanone	1,200	1,800	270	2,600	0
2, 3-Butandione	0	160	0	230	0
4-Methyl-2-pentanone	0	0	0	0	2
4-Pentene-2-one	0	0	0	0	70
Furan	0	3,900	1,500	16,000	120
Dihydrofuran	0	0	900	1,600	0
2-Methylfuran	5,500	670	1,200	6,100	0
3-Methylfuran	370	270	0	1,400	0
Ethylfuran	8,300	2	70	0	0
2, 5-Dimethylfuran	0	0	0	940	0
Ethylcyclobutanol*	0	0	>240,000	14,000	0
Cycloheptene*	0	0	>40,000	0	0
3-Cycloheptene-1-one*	0	0	>1 × 10 ⁶	>100,000	0
Ethenylcyclohexane	0	0	0	200,000	0

ND, not determined.

* Preliminary identification.

† Compounds unstable in the analysis or without appropriate standards are shown as >.

‡ Total includes both dimethylsulphide and acetone.

Levels of alkanes, butanal, ethylcyclobutanol and dimethylsulphide in *G. tikvahiae* decreased in going from nutrient-enriched to nutrient-deprived cultures. However, concentrations of furans, ketones and the remaining aldehydes all increased. These differences seem to reflect a change in metabolism in the two cultures. It is known that lipid levels in organisms change in response to nutrient availability¹⁶; however, such changes have been observed only with lipids containing more than 15 carbon atoms. Our results show that changes also occur in the low molecular weight ranges.

Several species of phytoplankton (microalgae), *Thalassiosira* sp., *Gymnodinium* sp., *Emiliania huxleyi* and *Skeltotenella costatum*, were analysed by the same methods as the macroalgae. Only one very volatile compound was detected which appeared to be a C₅ diene. This means that these phytoplankton cannot be directly contributing the compounds in Table 1 to sediments.

A variety of low molecular weight volatile alkanes, alkenes, ketones, aldehydes, furans, and sulphides have been found in macroalgae and the following conclusions may be made from their distribution: (1) Macroalgae are the probable source of some of these same compounds found in recent marine sediments. The low isopentane/*n*-pentane ratio of marine sediments could be partly caused by macroalgae. (2) Nutrient deprived macroalgae, such as *G. tikvahiae*, show a distinctly different volatile compound distribution from nutrient enriched algae. (3) Specific volatile compounds such as the cycloheptene and 3-cycloheptene-1-one in *G. tikvahiae* may be useful fingerprints in identification of algae. (4) Phytoplankton do not appear to be the source of volatile organic compounds in recent sediments.

This work was supported by the Oceanography Section of NSF (grant OCE 80-19508). This is Woods Hole contribution no. 5099.

Received 20 April; accepted 29 June 1982.

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High abundance of algal 24-ethylcholesterol in Antarctic lake sediment

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Sterols are one of the most widely distributed group of organic compounds in the environment, ranging in geological age from Recent to Cretaceous, and are commonly found in soils and contemporary lake and marine sediments^{1–11}. The major sterols found in lake sediments are stenols, cholest-5-en-3 β -ol (C₂₇), 24-methylcholesta-5,22-dien-3 β -ol (C₂₈), 24-methylcholest-5-en-3 β -ol (C₂₈), 24-ethylcholesta-5,22-dien-3 β -ol (C₂₉) and 24-ethylcholest-5-en-3 β -ol (24-ethylcholesterol, C₂₉) and stanols, 5 α -cholestan-3 β -ol (C₂₇), 24-methyl-5 α -cholestan-3 β -ol (C₂₈) and 24-ethyl-5 α -cholestan-3 β -ol (C₂₉) (refs. 5–8, 10, 11). C₂₉ sterols are abundant in vascular plants, whereas C₂₇ sterols are often dominant in plankton. Thus C₂₉/C₂₇ sterol ratios are believed to be indicators both of allochthonous and autochthonous contributions of organic matter for lakes and of terrigenous sources of organic matter in marine environments^{5,7,8,10,11}. In the Antarctic, vascular plants are only found in the Antarctic Peninsula¹². Thus the sterol composition of Antarctic lake sediments is expected to be considerably different from those in the temperate and tropical zones. Here we report the first analyses of sterols in Antarctic lake sediments, which are found to contain high abundances of 24-ethylcholesterol. Such sterols must arise from blue-green and green algae in lakes and surrounding soils.

During 1976–77 and 1980–81 austral summers, sediments, epibenthic algae and moss were collected from the dry valleys of Victoria Land in the Antarctic (Fig. 1, Table 1). Characteristics of the dry valley lakes are described elsewhere^{13–17}. The lakes and ponds studied are all saline. The primary productivity measurements in Lakes Vanda and Bonney yielded low values of 29 and 31 mg C m⁻² day⁻¹, respectively¹³. Lake and pond bottom sediment samples were taken with stainless steel scoop, with polyvinyl chloride pipe (length, 340 mm; i.d., 89 mm) by a diver, or using a Kitahara-type water sampler. The algae were collected from the lakeshore, which is usually covered with epibenthic algae. These samples were transferred to a glass bottle or wrapped in a Teflon sheet and stored at temperatures below 0°C until analysis. Wet samples (10–300 g) were first homogenized with an Ace Homogenizer (10⁴ r.p.m., Nihon Seiki Co. Ltd), saponified with 0.5 M potassium hydroxide in methanol solution (80°C, 2 h) and centrifuged (1,800 g). Supernatants and residues were separately acidified, extracted with

ethyl acetate after the addition of distilled water, and then concentrated under reduced pressure at temperatures below 30°C.

Part (1–1/10) of the ethyl acetate extracts was evaporated to dryness, dissolved in 50- μ l benzene: ethyl acetate (1:1) and chromatographed on a silica gel column (180 $\times$ 4 mm i.d., 100 mesh, 5% water). After elution of 2 column volumes of hexane, 3 column volumes of benzene: ethyl acetate (95:5) fractions were evaporated to dryness, dissolved in 100 μ l benzene and rechromatographed through a silica gel column impregnated with 3% potassium hydroxide (180 $\times$ 4 mm i.d., 100 mesh). Fractions eluted with 3 column volumes of benzene: ethyl ether (1:1) were concentrated and trimethylsilylated (TMS) with 25% N,O-bis(trimethylsilyl)acetamide in acetonitrile solution (1 h, room temperature). The TMS ethers of sterols were determined using a combined Shimadzu LKB 9000 gas chromatograph-mass spectrometer (GC-MS). The GC-MS conditions are given on Table 1 legend.

The individual sterols were identified by comparison of their retention times and mass spectra with those of authentic compounds. Stenols and stanols were quantified by measuring peak height on the mass fragmentogram carried at m/z 129 and 215 which are characteristic of the TMS ethers of Δ^5 -stenols and 5 α -stanols, respectively^{3,5,6,18}. The detection limits of the sterols by mass fragmentography were $\sim$ 0.1 ng. A blank run showed the presence of possible contaminants, but only minor amounts that would not affect the analytical results. To determine the analytical reliability, four replicate experiments using authentic compounds and a lake sediment were carried out and gave recoveries of cholest-5-en-3 β -ol, 24-methylcholest-5-en-3 β -ol and 24-ethylcholesterol of 83 (standard deviation, s.d., 11), 96 (s.d., 4.0) and 99% (s.d. 4.9%), respectively.

The contents of the six stenols and three stanols identified in the sediment samples range from 0.11 to 19 and 0.0 to 7.2 μ g per g dry sediment, respectively (Table 1). The amounts of sterols in the sediment from Don Juan Pond are extremely low, while those of Lakes Fryxell and Joyce are considerably higher. However, the amounts of stanols in the sediments from Lakes Vanda, Bonney and Joyce were extremely low. The ratios of sterol carbon to the total organic carbon contents differ considerably, ranging from 0.26 to 3.9 $\times$ 10⁻³, comparable with those of contemporary lake sediments from the temperate zone (0.27–5.2 $\times$ 10⁻³)^{7,8,11}.

The sources of stanols in contemporary lake sediments in the temperate zone have been widely discussed elsewhere^{4,6–8}. Our stanol composition should therefore be attributed to the hydrogenation of stenols, algal materials as shown in Table 1 and/or subsequent preferential degradation of stenols relative to stanols, as in the case of the sediments in the temperate zone.

Surprisingly 24-ethylcholesterol is the dominant sterol in all these sediment samples. It is also the most prominent sterol in some soils and water samples from the dry valley areas (G.M.,

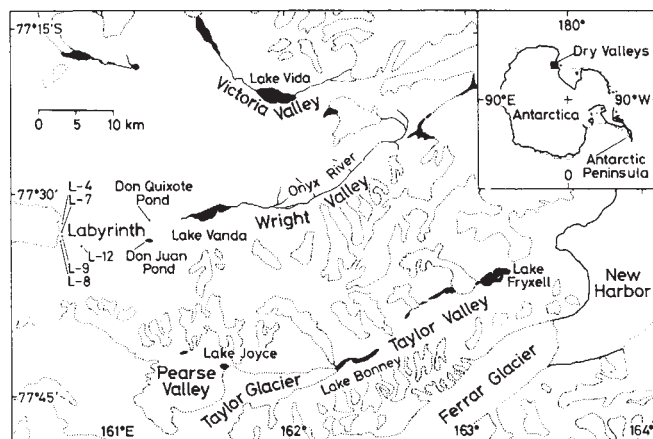


Fig. 1 Sampling locations in the dry valleys of Victoria Land in the Antarctic. L-4, L-7–L-9 and L-12 are unnamed ponds.

Table 1 Sterols found in sediments, epibenthic algae and moss from the dry valleys of Victoria Land in the Antarctic

Nature of sample¶	Sediment							Epibenthic algae				Moss
	Lake Vanda G, sd	Don Juan Pond DYB, sd	L-4 pond DYO, sd	L-8 pond RB, sd	Lake Fryxell B, fsd	Lake Bonney GO, st	Lake Joyce OG, sd	L-8 pond* O	L-12 pond† O	Lake Fryxell‡ OG	Lake Bonney§ OG	Fryxell Hut Br & SG
Stenols												
Concentration (µg per g) #	4.1	0.11	1.7	1.5	19	8.2	16	6.8	14	0.56	5.8	5.1
Composition (%)												
A	2.1	2.6	0.0	1.6	2.2	7.1	1.4	3.1	0.8	3.4	2.7	0.0
B	14.6	27.6	26.0	22.1	4.1	8.8	16.9	39.0	14.4	46.7	22.3	2.7
C	2.7	9.0	0.0	2.4	24.3	30.2	19.8	5.3	3.1	8.3	9.9	3.1
D	4.9	10.4	6.0	5.4	11.1	8.8	19.4	6.0	5.8	12.4	19.2	54.7
E	10.0	6.4	3.5	7.5	8.0	8.0	0.0	6.6	13.2	6.7	4.8	3.1
F	65.7	44.0	64.5	61.0	50.3	37.1	42.5	40.0	62.7	22.5	41.1	36.4
Stenols												
Concentration (µg per g) #	0.0	0.016	0.47	0.42	7.0	0.075	0.73	ND	0.68	0.0	0.20	0.079
Composition (%)												
G	—	44	90.0	84.4	11.0	51	42.3	—	13	—	30.8	56
H	—	44	2.1	6.0	45.9	49	5.9	—	10	—	26.4	0
I	—	12	7.9	9.6	43.1	0	51.8	—	77	—	42.8	44
Sterols-C**/TOC†† (×10 ³)	1.8	0.26	1.9	0.95	1.3	3.9	3.6	0.12	0.44	0.029	0.89	0.040
C ₂₉ /C ₂₇ ‡‡	4.5	1.4	1.4	1.5	7.2	2.7	2.2	1.1	5.1	0.60	1.8	11
Stenols/Stenols	0.0	0.24	0.28	0.28	0.37	0.091	0.043	—	0.049	0.0	0.034	0.015

GC-MS conditions: silanized glass column (200 cm × 3 mm i.d.) packed with 1% silicone OV-1 on 80–100 mesh Chromosorb W AW DMCS; temperature, injector 300 °C, column programmed from 230 to 290 °C at 5 °C min⁻¹, molecular separator 310 °C, ion source 330 °C; carrier gas, helium at 30 ml min⁻¹; accelerating voltage, 3.5 kV; multiplier gain, 3.

* Blue-green algae, *Synechococcus* spp., *Phormidium* spp. and *Oscillatoria* spp. being the main species. † Mainly *Phormidium* spp. ‡ Mainly *Oscillatoria* spp. § Mainly *Phormidium* spp. and diatom, *Hantzschia amphioxys*. || *Sarconeureum glaciale*. ¶ G, grey; sd, coarse and fine sands; DYO, dull yellowish brown; DYO, dull yellow orange; RB, reddish brown; B, black; fsd, fine sand; GO, greyish olive; st, silt; OG, olive grey; O, orange; Br & SG, brown and silver green. # Dry base. ND, not determined. ** Sterols as carbon. †† Total organic carbon, which was determined by dry combustion using a CHN analyser (Yanako MT2 CHN Coder) after treatment with 6 M hydrochloric acid to remove inorganic carbon. A: Cholesta-5,22-dien-3β-ol. B: Cholest-5-en-3β-ol. C: 24-Methylcholesta-5,22-dien-3β-ol. D: 24-Methyl-cholest-5-en-3β-ol. E: 24-Ethylcholesta-5,22-dien-3β-ol. F: 24-Ethylcholest-5-en-3β-ol. G: 5α-Cholestan-3β-ol. H: 24-Methyl-5α-cholestan-3β-ol. I: 24-Ethyl-5α-cholestan-3β-ol. ‡‡ E + F + I/A + B + G.

unpublished results). Thus the C₂₉/C₂₇ sterol ratios are high (1.4–7.2), and comparable with those of lake sediments from the temperate zone (0.48–8.5)^{6–8,10,11}, attributed to sterol inputs from vascular plants. However, no vascular plants are present in the dry valley areas. It is probable that there is preferential degradation in the sediments of C₂₇ relative to C₂₉ sterols, because the C₂₉/C₂₇ sterol ratios increase with increasing sediment depth in certain temperate lacustrine sediments^{6,8}. However, a core sediment taken from Lake Fryxell (20 cm long) showed that the vertical changes in the C₂₉/C₂₇ ratios are small (G.M., unpublished results). In addition the C₂₉/C₂₇ values of water samples through the depths from 5.4 to 65.9 m in Lake Vanda are very high (1.1–5.3, G.M. *et al.*, unpublished results). Furthermore incubation experiments both in water and sediment have not revealed the marked changes in C₂₉/C₂₇ ratios (ref. 8 and G.M., unpublished results). Thus the effect of preferential degradation of C₂₇ sterols relative to C₂₉ sterols in cold dry valley lakes is small. Therefore there seem to be at least three possible sources for these unusual distribution of sterols.

(1) An input of aeolian dust, because small amounts of pollen have been found in the sediment cores taken by Dry Valley Drilling Project from the dry valley areas¹⁹. In addition Aeolian dusts are believed to be the principal sources of biological lipids of terrigenous materials in open ocean environments^{20,21}. Normal alkanes are expected to be much more resistant to transformation than sterols in atmospheric transportation, because they are commonly found as the major constituents in atmospheric samples. However, the sterol/*n*-alkane ratios calculated for aerosols from the tropical North Pacific (<0.063–<0.50)²¹ and atmospheric fallouts from the Tokyo area (0.034 and 0.13)^{22,23} are significantly lower than those of the sediment samples (0.55–750) of the dry valley areas (ref. 24 and G.M., unpublished results). In addition cholest-5-en-3β-ol is the most dominant sterol in these aerosols and atmospheric fallouts.

These results showed that aeolian dust is not an important source for our sterols.

(2) Glacial erosion of sedimentary rocks, because much of the sedimentary organic carbon in the Ross Sea is derived from the rocks being eroded by glaciers on the Antarctic continent²⁵. Very long-chain *n*-alkanoic acids found in soils from the dry valley areas are apparently originated from sedimentary rocks of Precambrian to Cretaceous ages²⁶. Stanol/stenol ratios of sedimentary rocks can be expected to be much higher than the living organisms, but these ratios of our sediment samples are rather lower than the contemporary lake sediments from common temperate lakes^{4,6–8}. Further it was found that *n*-alkanes with the marked dominance of odd-carbon numbers and *n*-alkanoic acids with the great abundance of even-carbon numbers were not of ancient origin (ref. 24; G.M. unpublished results). Thus sedimentary rocks are also unlikely sources of our sterols.

(3) Biological sources, because many kinds of organisms, such as bacteria, fungi, blue-green and green algae, and mosses are found in the dry valley areas^{13,14,16,27}. Certain bacteria contain sterols, but none with 24-ethylcholesterol as the most abundant sterol^{28,29}. The principal sterol of fungi is ergosterol³⁰. Both organisms are therefore unlikely sources of our sterols. Moss contains 24-methylcholest-5-en-3β-ol and 24-ethylcholesterol as their dominant sterols (Table 1), but the distribution of mosses in the areas studied is restricted; they only occur around Lake Fryxell in Taylor Valley. Thus, mosses are also unlikely sources of the sterols in the sediments. 24-Ethylcholesterol as the major sterol has been found in some Chlorophyceae^{31–33}, Xanthophyceae³¹ and Rhodophyceae³¹. *Synura petersenii*³⁴ and *Phormidium luridum*³⁵ also contain 24-ethylcholesterol as the most dominant sterol. Our analytical results showed that 24-ethylcholesterol is the major sterols in epibenthic algae, mostly blue-green algae, which are widely distributed, collected from L-4 and L-12 ponds and Lake

Bonney. Therefore algae, including green and blue-green algae, especially *Phormidium* spp. are important sources of 24-ethylcholesterol in Antarctic dry valley lake sediments. In addition soil algae may contribute to the high abundances of C_{29} sterols in lake sediments²⁷.

We thank the Japan Polar Research Association, Antarctic Division, DSIR, New Zealand, US NSF and US Navy for logistic support, Drs S. Nakaya, T. Cho, E. Wada and G. M. Simons Jr and his research group for assistance in collecting samples, Professor H. Fukushima for the identification of algae and Dr H. Kanda for the identification of moss, and Drs K. Ogura and T. Itoh for useful suggestions.

Received 25 February; accepted 8 July 1982.

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Direct demonstration of fluid secretion by glomerular renal tubules in a marine teleost

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The evidence for fluid secretion by the kidneys of teleosts is indirect¹⁻⁴. By using *in vitro* microperfusion of renal tubules⁵, we have observed fluid secretion in the proximal tubule of the winter flounder *Pseudopleuronectes americanus*. Unlike the isosmotic fluid transfer across most vertebrate proximal tubules, the secreted fluid in this teleost could be hyperosmotic to the bathing medium. More importantly, fluid secretion was not dependent on the transport of Mg, S or organic ions⁴⁻⁶, but appeared to be driven by NaCl, the major solute in the secreted fluid. This mechanism of NaCl-dependent fluid secretion has been previously undetected because renal clearances showed the overall net reabsorption of NaCl and water^{4,7}.

P. americanus has a glomerular kidney containing 10,400 glomeruli on average and no aglomerular renal tubules have

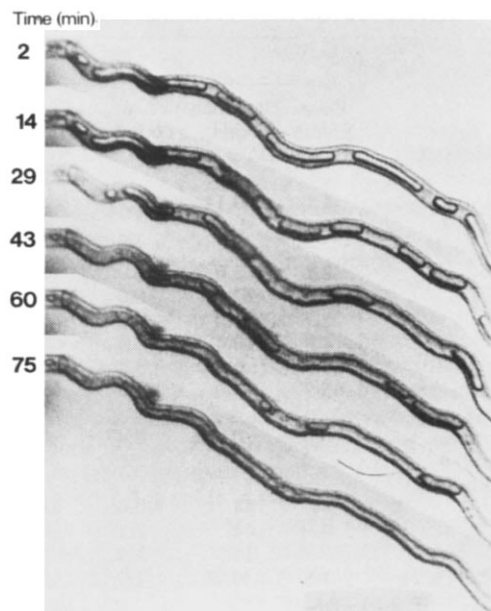


Fig. 1 Fluid secretion by proximal tubule. A 1.5-mm segment of a flounder proximal tubule (outer diameter 53 μ m, inner diameter 25 μ m) is shown in serial photographs. The tubule was held by two pipettes in aerated flounder Ringer's solution ($14 \pm 1^\circ\text{C}$) containing (mmol l^{-1}): NaCl 145, NaHCO_3 20, KCl 5, Na_2HPO_4 1.65, NaH_2PO_4 0.3, MgSO_4 1, CaCl_2 1, and glucose 10; pH 7.4. The left end of the tubule was cannulated with a perfusion pipette containing light paraffin oil; the right end of the tubule opened into a holding pipette as shown in Fig. 2. Perfusion filled the lumen with oil. When perfusion was stopped, epithelial fluid secretion fractionated the column of oil in the lumen and drove the flow of oil and secreted fluid towards the open end on the right. $\times 65$.

been observed⁸. Renal clearance studies do not reveal net secretion of fluid^{4,7}. However, isolated proximal tubules suspended in Ringer's solution between two glass pipettes and filled with oil can be observed to secrete fluid as in Fig. 1 where fluid secretion from bath to lumen displaced the oil from the lumen.

In subsequent studies of the characteristics of fluid secretion, the lumen was not filled with oil. Instead, one end of the tubule was closed by pulling it into a narrow glass pipette and the other end (open) was drawn into a collection pipette and sealed from the bath with uncured Sylgard, a viscous silicone resin (Fig. 2). In these experimental conditions secreted fluid flowed from the open end of the isolated proximal segments at rates averaging 27.1 ± 3.2 pl min⁻¹ mm tubule length ($\bar{x} \pm \text{s.e.}$, 19 collections, 12 tubules, 12 fish). Fluid secretion ranged from 9.8 to 56.6 pl min⁻¹ mm⁻¹ with no obvious correlation of secretion rate with the tubule's appearance. A conservative estimate for flounder proximal tubule length is 3 mm (ref. 8) which gives rates of *in vitro* fluid secretion (81 pl min⁻¹) and single nephron glomerular filtration (350-650 pl min⁻¹) within the same order of magnitude. For low filtration rates, proximal fluid secretion probably exceeds glomerular filtration as was observed in another glomerular flounder, *Paralichthys lethostigma*, in which urine flow rates were substantial (0.5 ml per kg per h) in the virtual absence of glomerular filtration (0.006 ml per kg per h)⁹.

The osmolalities of secreted fluid and the peritubular Ringer's were measured by freezing point depression (Clifton nanolitre osmometer) in nine experiments. In four experiments, secreted fluid was consistently isosmotic [292.1 ± 0.9 mOsm per kg H_2O ($\bar{x} \pm \text{s.e.}$), $n = 8$ collections] to the bathing medium (290.5 ± 3.4 mOsm kg H_2O , $n = 13$). In the other five proximal segments, secreted fluid was hyperosmotic (318.4 ± 4.6 mOsm per kg H_2O , $n = 10$) to the bathing medium ($P < 0.001$). The highest osmolality in secreted fluid was 346.8 mOsm per kg H_2O . These data are consistent with the existence of two functionally distinct segments in flounder proximal tubules, as are thought to occur in the kidneys of marine fish¹⁰. Bulger and Trump have identified a proximal tubule I and a proximal tubule II in fixed

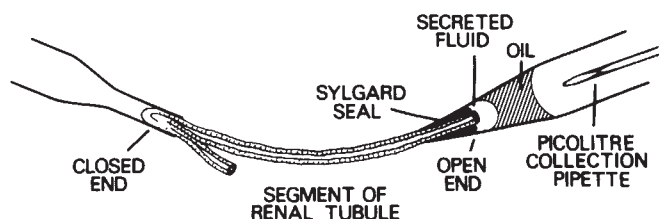


Fig. 2 Collection of secreted fluid. Uncured Sylgard 124 (Dow Chemical) was used as a seal between the secreted fluid and the bath. Secreted fluid flowing from the tubule accumulated under oil and was collected every 30–40 min for analysis. Isolated segments of proximal tubules were usually between 1.2 and 2.0 mm long. Fluid secretion in any one tubule was rather constant for the duration of the experiment, in one case for as long as 18 h.

renal slices of another flounder, the English sole¹¹. In fixed renal slices of the winter flounder we have also observed two histologically distinct segments of the proximal tubule (unpublished observations). However, in the present study it was not possible to distinguish proximal tubules I and II during dissection and all data were pooled to describe the *in vitro* function of the flounder proximal tubule in general (Fig. 3).

In 13 additional proximal tubule segments, the concentrations of Na, Cl, Mg and S (electron probe analysis measures the concentration of the element and not the ionic species) were measured in secreted fluid (SF), again in parallel with their corresponding concentrations in the peritubular bathing Ringer's (R)¹². Analysis of 22 SF/R sample pairs revealed that the Na concentration in SF was not significantly different from that in the bathing Ringer's; the 95% confidence interval of the Na SF/R ratio included unity (Fig. 3). In contrast, the concentrations of Cl, Mg and S (SO_4^{2-} is probably secreted) in secreted fluid were significantly greater than in the bathing Ringer's. The 95% confidence intervals of these SF/R ratios did not include unity, but they were large, consistent with varying capacities for concentration in different segments of the proximal tubule. The ability of flounder renal tubules to generate and maintain transepithelial gradients for water and ions is consistent with previous measurements of their transepithelial electrical resistance¹³ which is higher ($22.3\text{--}53.0\ \Omega\text{cm}^2$) than that usually found in mammalian renal proximal tubules ($4.9\text{--}5.7\ \Omega\text{cm}^2$)¹⁴.

Previously, when evidence for fluid secretion was obtained in mammalian renal tubules⁶ and teleostean kidneys^{2,4,15}, it was thought that the secretion of organic acids⁶ or divalent ions created an osmotic driving force^{2,4,15}. However, in the isolated renal tubules described here, the presence of organic acids or divalent ions in the bathing medium was not necessary to support fluid secretion. Organic acids were not present in the flounder Ringer's solution used, and in four additional experiments in which MgSO_4 was deleted from the bathing medium fluid, secretion continued at rates ($18.2 \pm 3.1\ \text{pl min}^{-1}\text{mm}^{-1}$) that were indistinguishable from control (*t*-test, sample means).

The replacement of bath Na^+ with choline, a large cation, immediately and completely inhibited fluid secretion ($n = 3$). Replacing choline-Ringer's with the original bathing medium (Na^+ -Ringer's) fully restored fluid secretion with the time course of the bath exchange ($<0.5\text{ min}$). In contrast, when Na^+ in the bath was replaced with Li^+ , a small cation, fluid secretion continued in all tubules studied ($n = 3$), but at progressively lower rates which paralleled the morphological deterioration of the tubule which occurred in Li^+ -Ringer's. Similarly, when Cl^- was replaced by a large anion (gluconate), fluid secretion immediately stopped ($n = 4$). Fluid secretion returned to control rates on providing the original conditions in the bath. However, when bath Cl^- was replaced with a small substitute (Br^-), fluid secretion continued at rates indistinguishable from the control ($n = 4$).

During this study some tubules were observed to constrict radially. The constrictions started as sharp invaginations of the basement membrane and extended radially towards the tubule

lumen. They occurred at single or multiple sites along the tubule. Some constrictions were sustained, others were periodic at about 3 cycles per minute. Peristalsis was not observed. Motion of axial cilia in the centre of the lumen was also occasionally seen. Solutions which stopped fluid secretion (Na^+ -free choline-Ringer's or Cl^- -free gluconate-Ringer's) also inhibited radial constrictions and ciliary motion. Solutions that restored fluid secretion (control bath) also restored tubule constrictions and ciliary motion. In tubules where radial constrictions and ciliary motion were not observed, the force of epithelial fluid secretion appeared to provide the hydrostatic pressure for axial flow. At present it is not clear whether myoepithelial cells or muscular layers^{10,16,17} are responsible for tubule constrictions. However, the constrictions were strong enough to move luminal fluid towards the open end of the tubule. Aiding axial flow in this manner might be a specialization of renal tubules that secrete fluid in glomerular kidneys having low filtration rates. In aglomerular kidneys radial constrictions might play an even greater part in the axial flow of luminal fluid.

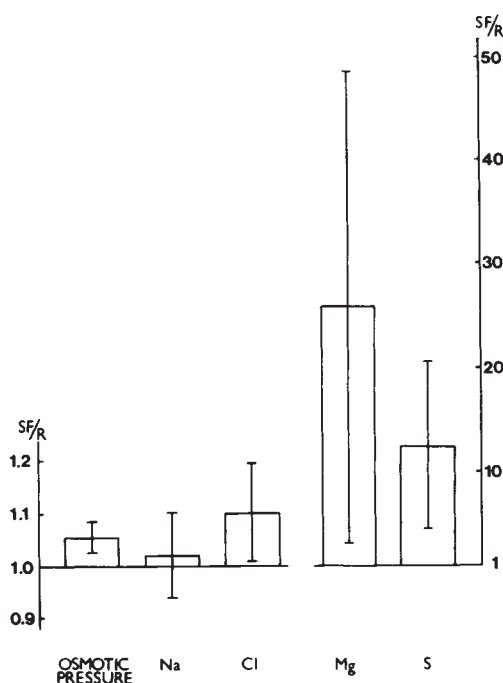


Fig. 3 Osmotic pressures and the concentrations in secreted fluid (SF) and peritubular Ringer's (R) expressed as the ratio SF/R. Mean values and their 95% confidence intervals are shown for two series of experiments. In the first, the osmotic pressure was measured by freezing point depression (Clifton Nanolitre Osmometer) in 18 SF and R sample pairs collected in experiments with nine proximal tubule segments. In the second, the concentrations of Na, Cl, Mg and S were measured in 22 SF/R sample pairs obtained in the study of 13 proximal tubule segments. Concentrations were measured in $100\text{--}200 \times 10^{-12}\text{ l}$ liquid droplets against appropriate standards (STD) of the same volume by electron probe analysis (X-ray wavelength dispersive spectroscopy, JEOL 733 Superprobe). Correlation coefficients of STD curves (chemical concentration versus X-ray counts) were high: for Na, 0.97 ± 0.02 , $n = 9$ STD curves; for Cl, 0.96 ± 0.02 , $n = 10$; for Mg, 0.994 ± 0.005 , $n = 11$; and for S, 0.998 ± 0.001 , $n = 11$. Deviations of STD curves from the origin ($\pm 3\text{ mM}$) gave rise to experimental error only in the measurement of concentrations close to zero, such as the known 1 mM Mg and S concentrations in the Ringer's. Some X-ray measurements gave values less than 0.3 mM for the known 1 mM MgSO_4 in the Ringer's. To prevent these measurements from increasing SF/R ratios, they were rounded up to 1 mM , but values greater than 1 mM were not rounded down, hence SF/R ratios for Mg and S are underestimates of the true ratios. All samples, SF, R and STD, were stored in Ringer's-equilibrated (alternatively: in light paraffin oil equilibrated with Ringer's) light paraffin oil until they were crystallized for electron probe analysis according to the method of Bonventre *et al.*¹². Evaporation into oil occurred at rates ranging from 0.4 to 0.9 pl min^{-1} for the $1\text{--}3\text{ nl}$ volumes handled in this study and introduced experimental error ($<12\%$). However, errors due to evaporation and sample dehydration were adjusted for by taking identical volumes of SF, R and STD of each tubule experiment together through all storage and dehydration steps. The use of more than one SF/R sample pair per tubule did not affect the statistical significance of the results.

The present study has provided the first collection and analysis of fluid secreted by a proximal tubule. Although vertebrate proximal tubules usually do not generate measurable transepithelial osmotic pressure differences¹⁸⁻²⁰ these results show that some proximal segments secrete hyperosmotic fluid, which may have a bearing on the previous *in vivo* observations of hyperosmotic urine in other marine teleosts^{2,21,22}. While the data confirm tubular secretion of Mg and S in marine fish, they do not support the hypothesis that fluid secretion is driven by Mg and S secretion alone⁴. A NaCl-dependent mechanism of fluid secretion is also present. This mechanism was unexpected because proximal fluid secretion in conjunction with glomerular filtration does not fit the present concept of hypo-osmotic regulation through the renal conservation of water.

This work was supported in part by NIH grant AM 26633 to K.W.B. and NSF grant DEB 7826821 and NIH grant S07-RR-05764 to the Mt Desert Island Biological Laboratory. I thank Dr B. Schmidt-Nielsen at MDIBL for the use of the Clifton nanolitre osmometer and Mrs Linda Phelps for secretarial assistance.

Received 12 January; accepted 21 June 1982.

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Absence of gonadotropin-releasing hormone receptors in human gonadal tissue

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The antifertility nature of the hypothalamic peptide gonadotropin releasing hormone (GnRH) and its synthetic agonist analogue (GnRH-A) has been repeatedly demonstrated in rodents, monkeys and humans (see ref. 1 for review). In all species studied, this action of GnRH was initially attributed to 'desensitization' of gonadal steroidogenesis due to loss of gonadotropin receptors in the testis and ovary, induced by high circulating levels of endogenous gonadotropins achieved following repeated administration of GnRH or GnRH-A²⁻⁵. Recently, an additional, direct inhibitory action of these peptides on ovarian granulosa^{6,7} and luteal⁸⁻¹⁰ and testicular Leydig¹¹⁻¹³ cells has been defined. This direct inhibition of gonadal steroidogenesis is mediated through specific high-affinity GnRH receptors in the respective target tissues^{8,9,12,14}. These receptors exhibit identical characteristics to GnRH receptors (GnRH-R)

in rodent anterior pituitary tissue^{9,12,14,15}. In the search for a naturally occurring ligand for gonadal GnRH-R, a biologically active 'GnRH-like' peptide has been isolated from rat ovary¹⁶ and testis^{17,18}, and from monkey testis interstitial fluid¹⁸. These findings suggest the production in rodent gonads of a GnRH-like peptide which, following interaction with gonadal GnRH-R, serves as a local modulator of steroidogenesis, and hence of reproductive capacity. If a similar situation exists in humans, GnRH-R should be detectable also in the human testis and ovary. We report here that we have been unable to detect any binding of radiolabelled GnRH-A to human corpus luteum or testis, or to the monkey testis. In contrast, GnRH-R were detected in adult and fetal human anterior pituitary tissue. Thus, we suggest that a direct inhibitory action of GnRH on primate reproductive function is unlikely. Furthermore, this important species difference highlights the need for a re-evaluation of certain rodent models designed to investigate primate, and especially human, reproductive physiology.

An area of considerable interest in reproductive biology concerns the apparently paradoxical antifertility nature of the natural hypothalamic-hypophysiotrophic hormone GnRH, responsible for the neural coordination of fertility. When administered to either laboratory animals or humans, either continuously or intermittently in high dosage, GnRH or its longer-acting agonist analogue (GnRH-A) interrupt normal cyclical ovarian activity, terminate established pregnancy, and inhibit testicular testosterone production and spermatogenesis (for review see ref. 1). In rodents, this inhibitory activity of GnRH is partly due to a direct action on ovarian and testicular steroidogenic cells. Recently, we and others (see ref. 14 for review) have reported that this extrapituitary action of GnRH and GnRH-A is mediated through specific receptor sites for the hormone either in the respective target cells, or in their cell membrane preparations.

To determine the presence of GnRH-R in primate gonadal tissue, we have used identical methods to those applied to measure rodent gonadal GnRH receptors. We used as the radioligand the iodinated GnRH agonist ¹²⁵I-GnRH-A¹⁴ which binds exclusively to high-affinity GnRH receptors in rat pituitary and gonads. Using this radioreceptor assay we have detected specific binding of the GnRH analogue to anterior pituitary membrane preparations from cadaver human glands (Table 1), despite the fact that these were removed 24-36 h postmortem at routine autopsy, and then stored at -70°C before the assay. As considerable loss of GnRH receptors may have occurred during the time from death to freezing of the tissue, the antemortem content of pituitary GnRH receptors was probably considerably greater. We have been unable to obtain any fresh normal human pituitary tissue, although two samples of freshly obtained human pituitary adenomatous tissue were examined, neither of which contained GnRH-R (Table 1). One of these was a mixed acidophil chromophobe adenoma from a patient with acromegaly (growth hormone-secreting tumour), while the other specimen was a chromophobe adenoma secreting large quantities of prolactin. GnRH receptors were also detectable in homogenates from single human fetal pituitary glands obtained either from prostaglandin-induced abortions or at hysterotomy (Table 1). In the case of the former specimens, intra-uterine fetal death occurred 12-20 h before sample collection. However, when rat pituitaries were either collected and snap-frozen immediately after decapitation, or analysed without previous freezing and thawing, the binding of the ¹²⁵I-GnRH-A was much greater (Table 1). As this radioreceptor assay allowed detection of GnRH-R in human pituitary tissue obtained many hours after death, we used it as a method for measuring GnRH-R in fresh human gonadal tissue obtained at operation and frozen immediately. Before the radioreceptor assay, all gonadal tissues were treated in an identical manner to the human pituitaries.

There was no specific uptake of ¹²⁵I-GnRH-A by membranes prepared from corpora lutea obtained at various stages from the mid- to late-luteal phase of the menstrual cycle from six

Table 1 ^{125}I -GnRH-A binding to human and rat anterior pituitary tissue

Tissue source	mg Protein per tube	c.p.m. Bound		Specific (Bo-NSB)	Total c.p.m. added (T)	SB/T ×100
		Bo	NSB			
Cadaver pit. (♀, 61 yrs)	1.2	1,191 ± 21(3)	788 ± 69(3)	403	16,412	2.46
Cadaver pit. (♀, 30 yrs)	1.4	5,030 ± 143(2)	4,173 ± 13(2)	856	47,809	1.79
Cadaver pit. (♂, 73 yrs)	1.1	4,867 ± 6(2)	4,098 ± 88(2)	769	47,809	1.61
Cadaver pit. (♀, 72 yrs)	1.2	1,027 ± 12(5)	829 ± 10(5)	198	7,955	2.49
Cadaver pit. (♀, 53 yrs)	0.76	797 ± 10(5)	667 ± 16(5)	129	7,955	1.62
Cadaver pit. (♂, 40 yrs)	1.1	1,001 ± 14(5)	752 ± 25(3)	249	9,118	2.73
Pituitary adenoma (acidophil) (♂, 42 yrs)	0.55	2,581 ± 113(2)	2,780 ± 269(2)	ND	47,809	—
Pituitary adenoma (prolactinoma) (♀, 56 yrs)	0.69	410 ± 11(3)	395 ± 31(2)	ND	7,955	—
Fetal pit. (♂, 18 weeks)*	0.19	1,045 ± 82(3)	833 ± 37(2)	217	16,412	1.32
Fetal pit. (♀, 17 weeks)*	0.154	1,355 ± 130(2)	740 ± 38(2)	615	16,412	3.75
Fetal pit. (♀, 18 weeks)*	0.135	909 ± 87(3)	528 ± 12(2)	381	15,525	2.45
Fetal pit. (♂, 14 weeks)*	0.29	726 ± 6(2)	422(1)	303	7,955	3.8
Fetal pit. (♀, 16 weeks)†	0.29	394 ± 6(2)	319 ± 11(2)	75	7,955	0.94
Rat pit.‡	0.092	7,925 ± 179(2)	1,655 ± 339(2)	6,270	47,809	13.11
Rat pit.§	0.21	4,724 ± 107(3)	856 ± 73(2)	3,868	16,412	23.6
Rat pit.§	0.165	4,511 ± 181(3)	1,113 ± 17(2)	3,398	15,525	21.9
Rat pit.‡	0.57	2,614 ± 48(2)	566 ± 26(2)	2,048	7,955	25.7
Rat pit.‡	0.59	1,777 ± 40(2)	515 ± 36(2)	1,261	7,955	15.86

Cadaver human pituitary (cadaver pit.) tissue was obtained, from routine autopsy for non-pituitary-related disease, between 24 and 36 h post mortem, and snap-frozen following separation of the neurohypophysis. Human pituitary adenoma tissue was obtained at hypophysectomy and immediately snap-frozen in liquid nitrogen (the predominant histological staining pattern is indicated). Human fetal pituitaries were obtained after prostaglandin-induced abortion (*), or hysterotomy (†) and snap-frozen as soon as they were removed from the abortions. Their sex and approximate gestational age are indicated in parentheses. Male rat pituitaries (Sprague-Dawley rats) were obtained immediately after decapitation and analysed without previous freezing (§), or were snap-frozen then thawed immediately before assay (‡). After snap-freezing all tissue was stored at -70°C for 1–4 weeks before assay; this does not result in any loss of rat pituitary GnRH-R¹⁴. Membrane preparations from the adult human pituitaries were prepared by homogenization (in an Ultra-Turax homogenizer) of the minced tissue in 10 mM Tris-HCl pH 7.5 containing 1 mM dithiothreitol (DTT). After initial centrifugation at 1,000g for 10 min (in a Sorval RCSB centrifuge), the membranes were precipitated from the supernatant by centrifugation at 25,000g for 30 min. The membrane pellet was re-homogenized in a small volume (1–2 ml) of 10 mM Tris-HCl and filtered through nylon gauze before use in the radioreceptor assay. All preparative procedures were performed at 4°C . Individual human fetal and rat pituitaries were homogenized, on ice, in 0.5 ml of 10 mM Tris-HCl and an aliquot of the unfractionated homogenate used for GnRH-R analysis. 100 μl of membrane or homogenate preparation were incubated with 100 μl of ^{125}I -GnRH-A ($1.3 \times 10^{-11}\text{ M}$) with (NSB, nonspecific binding) or without (total binding, Bo) a 1,000-fold excess of unlabelled GnRH-A in a total volume of 300 μl of assay buffer (10 mM Tris-HCl, 1 mM DTT, 0.1% bovine serum albumin, BSA) at 4°C for 90 min. The reaction was stopped by dilution (15-fold) with phosphate-buffered saline (PBS, pH 7.5) and the membrane fragments were separated (under vacuum) by filtration, through Whatman GF/C glass fibre filters, pre-soaked in 1% BSA to reduce nonspecific binding. After washing with 20 ml PBS, the filters were dried and counted in a γ -spectrometer with a counting efficiency of 60%. Specific binding (SB) = Bo - NSB and is expressed as a percentage (SB/T × 100) of total bindable counts added (maximum possible binding of ^{125}I GnRH-A = 50% of added c.p.m.). Samples having the same total c.p.m. were analysed in the same radioreceptor assay; a positive rat pituitary control was used in each assay. Values shown are the mean ± s.e.; the number of replicates is shown in parentheses. Differences between Bo and NSB counts for each sample were analysed by unpaired Student's *t*-test and were considered significant if $P < 0.05$. ND, $P > 0.05$. On this basis, all human cadaver pituitary samples exhibited specific ^{125}I -GnRH-A binding. The average minimum significant difference between Bo and NSB counts (defined as 2 s.d. below the mean of Bo replicates) was ~175 c.p.m.

Table 2 ^{125}I -GnRH-A binding to human and rat ovarian tissue

Tissue source	mg Protein per tube	c.p.m. Bound			Total c.p.m. added (T)	SB/T ×100
		Bo	NSB	Specific (Bo-NSB)		
<i>a</i>						
Human corpus luteum 1	3.38	458 ± 11(3)	447 ± 15(4)	ND	9,625	—
Human corpus luteum 2	2.5	631 ± 27(3)	519 ± 23(4)	ND	9,625	—
Human corpus luteum 3	2.4	830 ± 74(3)	774 ± 50(4)	ND	16,194	—
Human corpus luteum 4	2.6	890 ± 28(4)	842 ± 9(5)	ND	8,491	—
Human corpus luteum 5	1.62	783 ± 68(5)	822 ± 62(5)	ND	8,491	—
Human corpus luteum 6	1.55	568 ± 26(5)	558 ± 15(5)	ND	8,491	—
<i>b</i>						
Human corpus luteum 7	0.43	2,830 ± 242(5)	2,748 ± 185(5)	ND	15,271	—
Human corpus luteum 8	0.77	3,999 ± 248(6)	4,057 ± 168(6)	ND	33,185	—
Human corpus luteum 9	1.07	1,794 ± 62(6)	1,919 ± 48(6)	ND	17,501	—
Human corpus luteum 10	0.13	1,541 ± 25(3)	1,548 ± 134(3)	ND	20,214	—
<i>c</i>						
Rat ovaries 1 (random cycle)	2.57	1,444 ± 39(4)	577 ± 17(5)	867	9,118	9.50
Rat ovaries 2 (random cycle)	2.62	2,000 ± 55(5)	841 ± 18(5)	1,159	8,491	13.65
Rat ovaries 1 (luteinized)	2.98	1,304 ± 65(3)	528 ± 33(4)	776	9,625	8.06
Rat ovaries 2 (luteinized)	2.53	1,436 ± 56(3)	528 ± 33(4)	909	9,625	9.44
Rat ovaries 3 (luteinized)	1.3	1,721 ± 76(3)	647 ± 36(3)	1,073	16,194	6.6

a, Human corpora lutea were individually excised as discrete tissue, without surrounding ovarian stroma, during laparotomy for either tubal reconstructive surgery, hysterectomy for uterine myoma, or endometriosis. Corpora lutea were obtained from the mid-late luteal phase of the menstrual cycle; the patients had not received any hormonal treatment in the 6 months before surgery. Corpus luteum tissue was immediately rinsed in ice-cold PBS, pH 7.4 to remove any adherent blood clot, snap-frozen in liquid nitrogen without further processing, then stored at -70°C for 1–4 weeks before assay. When required for assay, ovarian membranes were prepared from the frozen tissue as described in Table 1 legend. **b**, As in **a** except that membranes were prepared from freshly obtained corpora lutea within 40 min of their removal from patients, without freezing or storage of the tissue, and assayed immediately. **c**, Rat ovaries were obtained from adult female animals at random stages of their oestrous cycles (random cycles) and from 25-day-old immature females treated with 50 IU of PMSG (pregnant mare serum gonadotropin; Organon) followed 5 days later by 25 IU of HCG and were killed 3 days later. These ovaries consist predominantly of luteinized follicles (luteinized ovaries). Rat ovarian tissue was frozen and thawed in the same way as for human tissue. Membrane preparations were prepared, incubated with ^{125}I -GnRH-A and the results expressed as described for Table 1. The common total c.p.m. indicates that all tissues were analysed in the same assay, each of which incorporated positive rat ovarian membrane and pituitary homogenate controls. The average minimum significant difference between Bo and NSB counts was ~170 c.p.m.

Table 3 ^{125}I -GnRH-A binding to human, monkey and rat testicular tissue

Tissue source	mg Protein per tube	c.p.m. Bound		Specific (Bo-NSB)	Total c.p.m. added (T)	SB/T $\times 100$
		Bo	NSB			
Human testis 1	2.18	1,185 $\pm$ 21(5)	1,141 $\pm$ 10(5)	ND	9,439	—
Human testis 2	3.46	929 $\pm$ 44(3)	987 $\pm$ 25(3)	ND	9,740	—
Human testis 3	3.55	951 $\pm$ 1(2)	974 $\pm$ 14(2)	ND	9,740	—
Human testis 4	5.87	922 $\pm$ 100(3)	838 $\pm$ 25(4)	ND	14,564	—
	2.96	943 $\pm$ 7(3)	961 $\pm$ 54(4)	ND	14,564	—
Human testis 5	4.4	873 $\pm$ 48(3)	838 $\pm$ 25(4)	ND	14,564	—
	2.31	854 $\pm$ 43(3)	960 $\pm$ 54(4)	ND	14,564	—
Monkey testis 1	2.0	348 $\pm$ 10(5)	334 $\pm$ 6(5)	ND	9,439	—
Monkey testis 2	1.63	538 $\pm$ 12(5)	573 $\pm$ 15(5)	ND	9,439	—
Monkey testis 3	4.40	313 $\pm$ 13(5)	283 $\pm$ 4(5)	ND	9,439	—
Rat testis 1	4.6	767 $\pm$ 12(5)	561 $\pm$ 16(5)	207	9,439	2.19
Rat testis 2	5.53	888 $\pm$ 62(3)	587 $\pm$ 13(3)	301	14,564	2.07
Rat testis 3	4.84	751 $\pm$ 39(3)	562 $\pm$ 9(3)	189	14,564	1.3
Rat testis 4	5.44	742 $\pm$ 55(6)	515 $\pm$ 28(3)	226	9,740	2.32

Human testes were obtained from 60–80-yr-old patients at orchidectomy representing primary treatment for prostatic carcinoma. None had received previous hormone therapy with oestrogen. Monkey (*Macaca fascicularis*) testes were obtained from healthy adult animals during orchidectomy for experimental purposes. The testes were immediately snap-frozen in liquid nitrogen, without processing, and stored at -70°C for 1–4 weeks before membrane preparation and analysis. Testes from adult rats were frozen and stored in the same way as the primate specimens. Membrane preparations were prepared and incubated with ^{125}I -GnRH-A as described for Table 1. The average minimum detectable difference between Bo and NSB counts was ~ 130 c.p.m. (2 s.d. less than the mean Bo).

individuals (Table 2), despite the presence of more membrane protein than for human cadaver anterior pituitary tissue. Fresh, unfrozen human corpora lutea analysed immediately after removal from the patients, were also devoid of GnRH receptors (Table 2). Thus, it is unlikely that the procedure of freezing and storage destroyed GnRH-R. Additionally, membrane preparations from whole, pre-menopausal human ovaries were devoid of GnRH receptors (data not shown), while identical membrane preparations from either fully luteinized rat ovaries or ovaries obtained from rats at random stages of their oestrous cycles contained many GnRH receptors. In each assay containing human ovarian tissue, at least one rat ovarian preparation and one rat pituitary homogenate preparation were included as positive controls. That the human corpus luteum membranes contained steroidogenic tissue was indicated by the presence of luteinizing hormone (LH) receptors, identified by binding of ^{125}I -HCG (human chorionic gonadotropin), in an aliquot of the preparation (data not shown).

Human and monkey testis membrane preparations also did not appear to interact with the labelled GnRH analogue (Table 3). Five different testicular preparations, obtained at orchidectomy performed as primary treatment for prostatic carcinoma, were all negative (Table 3). None of these patients had received previous hormonal (oestrogen) therapy. Similarly, no ^{125}I -GnRH-A binding was demonstrable in testis membrane preparations from three adult monkeys, obtained at orchidectomy (Table 3). Because the primate tissue had been rapidly frozen on removal, no attempt was made to separate the seminiferous tubules, which have no GnRH receptors, from the interstitial tissue, which in the rat is the only compartment exhibiting GnRH-R¹⁹. However, in one experiment in which fresh human testis was enzymatically dispersed to yield predominantly Leydig cells, there was no detectable GnRH binding, while an identical interstitial cell preparation from fresh rat testes was positive. In addition, the four rat testis membrane preparations, which were positive for GnRH-R (Table 3) comprised both tubular and interstitial cell elements as they had been stored and were prepared in an identical manner to the human samples. As with the human corpora lutea, human and monkey membrane preparations contained Leydig cell elements as evidenced by the presence of ^{125}I -HCG binding.

Thus, we have been unable to demonstrate the presence of any GnRH receptors in human corpora lutea using the most sensitive method available for detection of GnRH-R in pituitary and gonadal tissues. It is possible that the concentration of GnRH-R in human gonads is so low as to be unmeasurable by these techniques, but this seems unlikely. Indeed, using very similar methodology, Asch *et al.*²⁰ were unable to detect any GnRH analogue binding in monkey corpora lutea. Further-

more, they failed to show any effect of a GnRH agonist on progesterone production by acutely dispersed monkey luteal cells *in vitro*²¹. Similarly, human granulosa cell steroidogenesis *in vitro* was unaffected by either GnRH or an agonist analogue²². Although there has been one report²³ of direct inhibition of progesterone production for human granulosa cultured in the presence of a GnRH agonist analogue, this did not occur until after 96 h in culture. Further, the granulosa cells were exposed to concentrations of exogenous gonadotropin higher than that to which they would be exposed *in vivo* and this might alter their steroidogenic response pattern. In contrast, the inhibitory effects of GnRH peptides on steroidogenesis are observed immediately when rat luteal cells^{8,10}, rat granulosa cells^{6,7}, and porcine granulosa cells²⁴ are similarly incubated with GnRH or GnRH-A *in vitro*. Therefore, in the human and non-human primate it seems unlikely that the antifertility effect of this group of peptides involves a direct gonadal site of action. A similar conclusion might also apply to the mouse, as in this species no GnRH effect on Leydig cell function can be demonstrated *in vitro* and receptors for this peptide are also absent²⁵. It is possible that the laboratory rat is not representative of other species in respect of the direct gonadal effect of GnRH. Hence data from such studies in rats may not be applicable to primates. Specifically, we infer, from the data reported here, that human gonadal tissue may not produce any 'GnRH-like' material acting as an intragonadal modulator of reproductive function.

We thank the gynaecologists in Birmingham, Oulu and Helsinki for providing us with human ovarian tissue, and the various surgeons for providing the human testis specimens. This work was supported by grants from the MRC (to R.N.C.), the Finnish Academy (to I.T.H.) and the British Council Academic Links and Interchange Scheme (R.N.C. and I.T.H.).

Received 2 March; accepted 7 July 1982.

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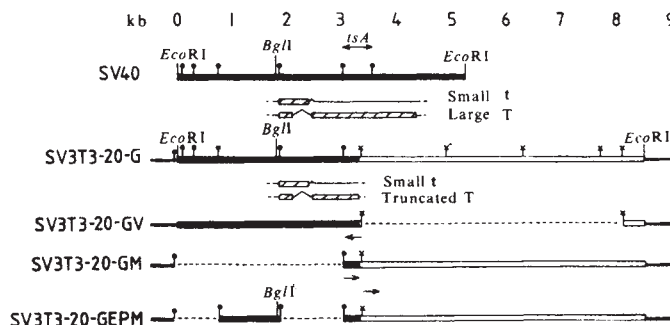


Fig. 1 Structures of pSV3T3-20-G and recombinants derived from it. At the top is shown the restriction map of SV40 DNA², linearized at the single *EcoRI* site. *HindIII* sites are marked †; the double-headed arrow indicates the *HindIII* fragment in which most *tsA* mutations map²². Also shown are the structures of the two early mRNAs found in productively infected permissive cells². —, Translated sequences; —, untranslated sequences; ^, splices. The map of SV3T3-20-G is adapted from ref. 3; *XbaI* sites are marked x. —, SV40 DNA; —, mouse DNA; —, vector (pAT153) DNA. The two RNAs shown below are transcribed from the SV40 early promoter of this template (ref. 27 and M.L. and P.W.J.R., unpublished data). pSV3T3-20-GV and pSV3T3-20-GM were constructed by digesting pSV3T3-20-G to completion with *XbaI* and *HindIII* respectively, religating the digested DNA and transforming³ it into *Escherichia coli* HB101. pSV3T3-20-GEPM was constructed by inserting the *HindIII*-C fragment of SV40 DNA into the unique *HindIII* site of pSV3T3-20-GM. The DNA sequence of the virus-host junction was determined^{27,40} by sequencing from the *HindIII* and *XbaI* sites as indicated by the arrows.

A fragment of the SV40 large T-antigen gene transforms

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SV40-transformed cells retain and express all the sequences encoding large T antigen¹; this protein, in its entirety, appears to be essential for transformation^{1,2}. We have isolated³ a segment of integrated viral DNA (SV3T3-20-G) which encodes only the N-terminal half of T antigen but is capable of transforming rat fibroblasts. SV3T3-20-G-transformed cells synthesize a truncated T antigen of apparent molecular weight (MW) 48,000. The sequence of the virus-host junction shows that this protein is partially encoded by the flanking mouse DNA and suggests that the SV40 DNA has integrated next to a mouse gene. The mouse DNA segment of SV3T3-20-G is not required for transformation although transcription of these flanking sequences may be activated by the insertion of viral DNA.

The SV40 early transcription unit is transcribed in productively infected permissive cells into two mRNAs with identical 5' and 3' termini but different splices² (Fig. 1). The larger, 2.6 kilobase (kb) mRNA encodes small t antigen, a protein of apparent MW 17,000 which is not essential for transformation⁴⁻⁶ but which may, in some circumstances, have an enhancing role^{1,7,8}. The smaller, 2.3 kb mRNA encodes large T antigen, a protein of apparent MW 94,000 which is found predominantly in the nuclei of transformed cells and is essential for transformation^{1,2}. This protein has many functions⁹⁻²¹ but it is not clear which of its functions is directly involved in the transformation of non-permissive cells.

SV3T3-20-G is a segment of integrated viral DNA cloned from the genome of the SV3T3 C120 line of SV40-transformed BALB/c 3T3 cells³. This 8.5 kb *EcoRI* fragment (Fig. 1) contains 3.3 kb of viral DNA and 5.2 kb of mouse DNA, which includes a sequence element present many times in the mouse genome (P. Brickell, D. S. Latchman and P. W. J. R., unpublished data). The viral DNA segment extends from the *EcoRI* site (nucleotide 1,782; according to the system of ref. 2) through the 5' half of the late transcription unit and the origin of DNA replication and into the 5' half of the early transcription unit. The viral DNA is interrupted by a junction with cellular DNA located at the beginning of the region in which the *tsA* mutations map²². It thus lacks many of the viral sequences thought to be essential for transformation^{1,2}. We were therefore surprised to find that when pSV3T3-20-G (the SV3T3-20-G *EcoRI* fragment cloned into the *EcoRI* site of pAT153) was introduced into rat-1 cells by calcium phosphate-mediated DNA transfection^{23,24}, foci of morphologically transformed cells arose (Fig. 2) at a frequency of 5 per μ g DNA per 10^6 cells, a frequency ~1% of that obtained with intact SV40 DNA²³.

Foci induced by pSV3T3-20-G appear about 10 days after those induced by the complete SV40 early region and are morphologically quite distinct, being flatter and less clearly delineated. Cells cloned from such foci are able to overgrow a monolayer of normal cells, have a more rounded morphology than the parental fibroblasts and multiply rapidly in medium containing 2% (v/v) fetal calf serum. This type of transformation has no precedent and we therefore examined the SV3T3-20-G DNA for unusual properties, first by determining the DNA sequence across the virus-host junction.

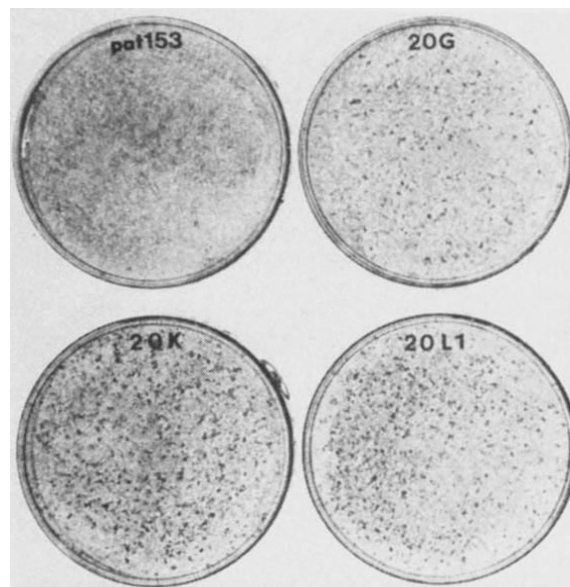


Fig. 2 Transformation by pSV3T3-20-G. Plasmid DNA (40 μ g) was transfected into confluent monolayers of rat-1 cells grown on 50-mm dishes using a slight modification²³ of the calcium phosphate co-precipitation technique²⁴. Cells were maintained as monolayers in Dulbecco modified Eagle's medium supplemented with 5% (v/v) fetal calf serum and antibiotics. The dishes were fixed and stained²³ 30 days after transfection. Cells were transfected with vector (pAT153) DNA, pSV3T3-20-G and two other recombinant plasmids (pSV3T3-20-K and pSV3T3-20-L1) which contain tandem duplications in the viral early transcription unit and which transform as well as, if not better than, wild-type SV40 DNA²³.

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We constructed subclones containing the virus-host junction, together with either the entire viral (pSV3T3-20-GV) or mouse chromosomal (pSV3T3-20-GM) segments, and determined the junction sequence as indicated in Fig. 1. The sequence obtained shows several interesting features (Fig. 3). An mRNA transcribed from the SV40 early promoter of SV3T3-20-G and containing the large T-antigen splice could be translated in the large T reading frame for 23 nucleotides beyond the virus-host junction, which occurs at SV40 nucleotide 3,713. Such a transcript would encode a truncated T antigen of calculated MW 45,000, the seven C-terminal amino acids of which are encoded by the flanking mouse DNA. Ten nucleotides beyond the translational stop codon is the sequence TATAAA which is one base removed from the canonical polyadenylation signal AATAAA^{25,26}. Transcripts of the size predicted if polyadenylation occurs at this site are found in SV3T3 C120 (ref. 27 and M.L. and P.W.J.R., unpublished data) and in cells transformed by pSV3T3-20-G (data not shown). In addition, cells transformed by pSV3T3-20-G contain the predicted truncated T antigen, which has an apparent MW of 48,000 (Fig. 4) and is also found in SV3T3 C120 (ref. 27). This protein may therefore be capable of inducing transformation.

An alternative explanation for our data was that the integration of the SV40 DNA had activated the adjacent DNA which contains a cellular transforming gene, a possibility strengthened by the sequence of the flanking mouse DNA (Fig. 3). Close to the virus-host junction there is a perfect consensus 'TATA' box, a feature of the 5' ends of eukaryotic RNA polymerase II transcription units²⁸. A sequence closely related to the consensus mRNA cap site²⁸ is located 34 nucleotides downstream of the first T of the 'TATA' box and this is followed, 42 nucleotides from what would be the initiating nucleotide, by an ATG triplet which marks the beginning of an open reading frame. It thus appears possible that the SV40 DNA has integrated next to a cellular gene. Neither we (D.M. and P.W.J.R., unpublished data) nor others^{29,30} have observed anything similar in other SV40-host DNA junctions.

We therefore attempted to transform cells with the viral and cellular segments in isolation. We also constructed an additional plasmid, pSV3T3-20-GEPM (Fig. 1), in which the segment of

T	Arg	Phe	Asn	Asp	Leu	Tyr	Pro	Gln	Ser	Thr	
5'	AGA	TTT	AAT	GAT	CTT	TAT	CCC	GAA	AGC	ACC	
3'	TCT	AAA	TTA	CTA	GAA	ATA	GGG	CTT	TCG	TGG	
	↑					↑					
	3,728					3,713					
	Val	Leu	Stop								
	GAG	CTT	TGA	CCA	GCT	GGC	TAT	AAA	ATG	GAG	
	CTC	GAA	ACT	GGT	CGA	CCG	ATA	TTT	TAC	CTC	
	GCT	TCC	CCC	ATC	TCT	CTC	TGG	TCC	GCA	AAT	
	CGA	AGG	GGG	TAG	AGA	GAG	ACC	AGG	CGT	TTA	
	ACT	CTA	GAC	CGC	GCT	CGC	TCA	TCT	CTC	AGC	
	TGA	GAT	CTG	GCG	CGA	GCG	AGT	AGA	GAG	TCG	
	Met	Gly	Arg	Cys	Ala	Ser	Leu				
	ACG	CTG	AC	ATG	GGC	AGG	TGC	GCT	AGC	CT	3'
	TGC	GAC	TG	TAC	CCG	TCC	ACG	CGA	TCG	GA	5'

Fig. 3 Sequence of the virus-host junction in SV3T3-20-G. Plasmid DNAs were cleaved with *Hind*III or *Xba*I, as shown in Fig. 1, and the resulting fragments were labelled at their 3' termini by incubation with reverse transcriptase and [α -³²P]dGTP or [α -³²P]dGTP²⁷. Sequencing was performed by the procedures of Maxam and Gilbert⁴⁰. Italicized nucleotides indicate host DNA. The numbers indicate SV40 nucleotide numbers.



Fig. 4 SV40 T antigens in rat cells transformed by pSV3T3-20-G. r20G7 (a rat-1 cell line transformed by pSV3T3-20-G) cells were labelled with ³⁵S-methionine and T antigens were extracted, immunoprecipitated and analysed by SDS-polyacrylamide gel electrophoresis as previously described²³. N, immunoprecipitation with normal serum; T, immunoprecipitation with anti-T serum.

SV40 DNA containing the promoter for the early transcription unit^{31,32} and the 72 base pair repeats which enhance the expression of linked genes³³ was inserted upstream of the cellular DNA, in case these sequences are necessary for the transcription of the mouse DNA. However, neither pSV3T3-20-GEPM nor pSV3T3-20-GM was capable of transforming rat-1 cells, although in the same experiment pSV3T3-20-G transformed at the expected frequency. In contrast, pSV3T3-20-GV induced foci, but at one-fifth the frequency of pSV3T3-20-G. Thus the truncated T antigen can transform even though it only contains the N-terminal half of large T antigen. The decreased efficiency with which pSV3T3-20-GV transforms relative to pSV3T3-20-G may be due to the fact that although pSV3T3-20-GV retains the hexanucleotide it lacks downstream mouse sequences which could play a part in efficient polyadenylation.

Despite the inability of pSV3T3-20-GM to transform, we have, because the sequence data suggest the presence of a mouse gene in the flanking DNA, searched for transcripts homologous to this segment in rat-1 cells and in SV3T3-20-G-transformed cells. Transfer hybridization experiments show that the transformed cells contain an increased level of RNA which can hybridize to an exclusively cellular fragment of SV3T3-20-G. Moreover, two-dimensional S₁ endonuclease mapping experiments have revealed such transcripts in SV3T3 C120, but not in other SV40-transformed mouse cells or in untransformed BALB/c 3T3 cells. The repeated sequence element in SV3T3-20-G complicates such analyses, however, and means that we cannot be certain that the RNAs are transcribed from SV3T3-20-G. We have now obtained subclones containing, separately, the repeated and unique segments of SV3T3-20-G and are analysing the transcripts in detail.

It seems unlikely that pSV3T3-20-G transforms through the action of small t antigen. Although small t antigen can have various roles during transformation by wild-type virions^{1,2,7,8}, extensive data show that this protein is not required for transformation^{1,2,4-6} and stable transformants containing or induced by small t antigen alone have not been reported. The alternative hypothesis is that transformation can be induced by the truncated T antigen. Previous attempts to transform cells with segments of SV40 DNA containing less than the whole early region have failed³⁴⁻³⁶. In these experiments, however, the

transfected DNA lacked both the termination codon for large T antigen and the polyadenylation site, so expression of a large T antigen-related protein could only occur if the integration event served to restore these control signals, a process which is certain to be extremely inefficient. In SV3T3-20-G the virus-host fusion required has already occurred.

When stained³⁷ for SV40 T antigen using the monoclonal antibody PAb419, which recognizes a determinant common to small and large T antigens³⁸, cells transformed by pSV3T3-20-G stain weakly in both nucleus and cytoplasm. The lack of nuclear retention suggests that the truncated T antigen may have a reduced ability to bind to DNA. It clearly does not bind to the cell-coded p53 protein (Fig. 4).

Colby and Shenk have recently shown³⁹ that SV40 genomes containing deletions which remove sequences coding for the C-terminal half of large T antigen, including the termination codon but not the polyadenylation signal, can immortalize rat embryo cells. Some of the cell lines thus generated have a fully transformed phenotype. The portion of the T antigen gene contained in their constructs is almost identical to that in SV3T3-20-G. However, their cell lines contain unstable T antigens whereas that encoded by SV3T3-20-G seems to be stable, a difference which may be due to the absence of the termination codon in their mutants. More surprising is the observation that their cell lines contain high levels of p53 whereas cells transformed by pSV3T3-20-G do not. These two sets of data clearly establish that the N-terminal half of large T antigen can mediate both immortalization and transformation. Our assay system is different from that used by Colby and Shenk and further studies are therefore required to explain the differences between the two sets of results.

The protein encoded by SV3T3-20-GV may transform through a hitherto unsuspected and novel property of large T antigen, perhaps by a mechanism quite distinct from that used by the intact protein. This idea is consistent with the fact that foci induced by pSV3T3-20-G are clearly distinguishable from those induced by intact SV40 DNA. The structure of SV3T3-20-G and our preliminary transcription data also raise the possibility that the integration of SV40 DNA may lead to the activation of adjacent cellular sequences.

We thank Wendy Colby and Tom Shenk for communicating unpublished data. D. M. holds a MRC research studentship and P. W. J. R. a Career Development Award from the CRC which also paid for this work.

Received 9 March; accepted 24 June 1982.

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***onc* gene amplification in promyelocytic leukaemia cell line HL-60 and primary leukaemic cells of the same patient**

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Cellular *onc* genes are a group of evolutionarily conserved sequences which are homologous to the transforming genes (*v-onc*) of oncogenic retroviruses¹. Their function in normal cells is not yet known, but the sequence homology between viral and cellular *onc* genes is consistent with the idea that neoplastic transformation may, in some cases, be due to increased levels of cellular *onc* gene expression. The cellular homologue, *c-myc*, of the transforming gene of avian myelocytomatosis virus (MC29)^{1,2} is involved in the pathogenesis of chicken B-cell lymphomas induced by the non-acute leukemia virus (RAV-2)³⁻⁶, and in these tumours, *c-myc* expression is enhanced by the nearby integration of the RAV-2 terminal repeat region³⁻⁶. Transcripts from the *c-myc* gene are detectable in a variety of human cells^{7,8}, and increased levels of *myc* mRNA have been occasionally detected in some neoplastic tissues^{7,8}. The highest levels have been detected in the cell line HL-60 (ref. 8) derived from neoplastic cells from a patient with acute promyelocytic leukaemia (APL)⁹. We have now investigated whether any structural alteration at the genomic level could account for the increased expression of *c-myc* in HL-60 and report here that the *c-myc* gene is stably amplified in HL-60 DNA. Amplification was also detected in primary uncultured leukaemic cells from the same individual, suggesting that the *c-myc* amplification may have been involved in the leukaemic transformation in this case.

The genomic organization of human *c-myc* sequences has recently been studied in our laboratory¹⁰. Isolation and characterization of several recombinant phages from a human DNA library suggested the existence of at least one complete gene and several partially related sequences which may represent pseudogenes¹⁰. The complete *c-myc* gene spans ~4.5 kilobases (kb) of the human genome where two *v-myc* homologous exons are interrupted by one intron¹⁰. Three putative pseudogenes, each of which contains ~0.3 kb of *c-myc*-related sequences and lacks 3' and 5' regions of the gene, have been characterized¹⁰. Northern blotting hybridization experiments using a *v-myc* probe revealed a 2.7 kb mRNA transcript in a wide variety of human haematopoietic and nonhaematopoietic cells, including normal fibroblast and normal peripheral blood lymphocytes^{7,8}. In most of the cells, *myc* mRNA was present in 1-10 copies per cell⁸. However, at least a 10-fold increase in mRNA was observed in a few cases, including one sarcoma, two carcinomas and two haematopoietic cell lines⁸. The highest levels of *myc* RNA were present in the human promyelocytic leukaemia cell line HL-60 (ref. 8). This cell line has the capability to differentiate into mature nonproliferating granulocytes on induction with several chemical agents, including dimethyl sulphoxide (DMSO)¹¹ and retinoic acid¹². *myc* transcripts were virtually undetectable in terminally differentiated HL-60 (ref. 8). To investigate whether any structural alteration of the *c-myc* gene or adjacent regulatory regions could account for the increased *c-myc* expression in HL-60, we have now analysed the structure of *c-myc* sequences in these cells by Southern blot hybridization¹³. Our study included DNA from the HL-60 cell line,

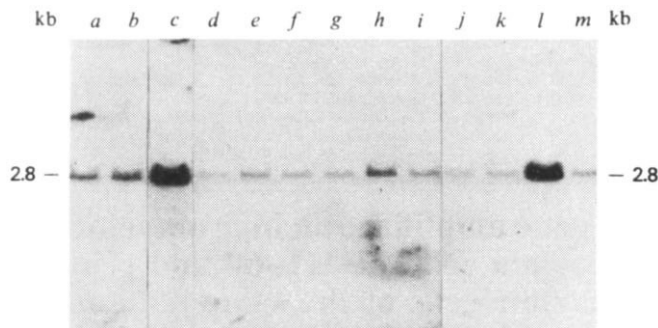


Fig. 1 Amplification of *c-myc* sequences in HL-60 DNA. DNA was extracted as previously described²⁴ from: a, peripheral blood ALL cells; b, peripheral blood CLL cells; c, HL-60 passage 70; d, peripheral blood APL cells; e, normal human spleen; f, Molt 4 cell line; g, human breast carcinoma cell line; h, peripheral blood APL cells; i, peripheral blood AMML cells; j, peripheral blood APL cells; k, peripheral blood CML cells, partial digestion; l, uncultured HL-60 cells; m, normal human peripheral blood lymphocytes; and 20 µg were digested with *Sst*I restriction endonuclease in standard conditions recommended by the supplier. Fragments were separated by electrophoresis on a 0.8% agarose gel. DNA was denatured, transferred to nitrocellulose as described by Southern¹³ and hybridization was performed using dextran sulphate²⁵. The probe was 2×10^6 c.p.m. of nick-translated pMC41-3RC DNA. pMC41-3RC is a recombinant pBR322 plasmid derived from λLMC41, the recombinant Charon 4A phage containing the human *c-myc* gene¹⁰. For subcloning into the plasmid, a 1.4 kb *ClqI*-*Eco*RI fragment containing the *c-myc* 3' exon¹⁰ was isolated on preparative agarose gel and ligated into *ClqI*-*Eco*RI-cleaved pBR322 (ref. 26).

from primary leukaemic cells of the same individual, from normal human tissues (peripheral blood lymphocytes and spleen) and different neoplastic cells. The latter were obtained from peripheral blood of patients with acute myelogenous leukaemia (AML; 3 cases), chronic myelogenous leukaemia (CML; two cases), APL (three cases), acute lymphocytic leukaemia (ALL; one case), chronic myelogenous leukaemia (CML; one case), acute myelomonoblastic leukaemia (AMML; one case) and from cell lines representing myeloid (KG-1 and K562; refs 14, 15 respectively) and lymphoid (Molt 4)¹⁶ leukaemia (AML; three cases), chronic myelogenous leukaemia endonuclease and hybridized to a probe (pMC41-3RC) derived from the 3' exon of the human *c-myc* gene (see Fig. 1 legend for details). This probe hybridizes to a single 2.8 kb *Sst*I fragment in human DNA and does not hybridize to any of the putative pseudogenes because they are homologous only to the 5' exon of the *c-myc* gene¹⁰.

Representative results shown in Fig. 1 indicate the intensity of the 2.8 kb band was approximately identical in DNA from all samples except the HL-60 cell line and the patient's original uncultured cells, in which cases the intensity was markedly increased. Similar results were obtained with DNAs from different passages of the HL-60 cell line and in differentiated cells after induction with DMSO (data not shown). These data suggest that the *c-myc* gene copy number is increased in HL-60 cells. To investigate the extent of amplification of *c-myc* sequences in the HL-60 genome we hybridized *Eco*RI digests of DNA from primary and cultured HL-60 cells to a probe (pMCO) containing the entire *v-myc* gene¹⁰. This probe detects all *c-myc* related sequences in the human genome, including the putative pseudogenes¹⁰. Figure 2 shows that the intensity of a 12.8 kb band, which corresponds to the entire functional gene, is markedly increased in the two HL-60 samples, confirming the results shown in Fig. 1. In contrast, the intensity of the 5.8 kb band, which corresponds to one of the *c-myc* putative pseudogenes, is uniform in all the samples, suggesting that the functional gene, but not the other related sequences, is amplified. Moreover, this band serves as an internal control, proving that the difference in intensity of the 12.8 kb band is not due to experimental artefacts such as irregular DNA transfer or non-uniform hybridization across the nitrocellulose filter. In addition, a 4.6 kb fragment appears in the two HL-60 DNA samples but not in the other samples. The origin of this fragment is unknown. We suggest that it could represent either an

unidentified pseudogene which would be part of the amplification unit or a new fragment generated during the *c-myc* amplification event in HL-60 cells. Finally, experiments were performed to quantify the amplification of *c-myc* in HL-60. Thirty µg of *Sst*I-digested HL-60 DNA were sequentially diluted (see Fig. 3) and the intensity of the hybridization band was compared with that obtained with 30 µg of normal human lymphocyte DNA. Hybridization to a probe derived from another human *onc* gene, *c-sis*, which is a single copy gene¹⁷, was used as a control in the same experiment. Whereas the intensity of the *c-sis* band is comparable in normal spleen and undiluted HL-60 DNA, a 16- to 32-fold dilution is necessary to bring the *c-myc* band to the same levels as the control DNA. These data indicate that the *c-myc* gene is amplified 16- to 32-fold in HL-60. Similar estimates were obtained by direct densitometric screening of X-ray films from experiments shown in Fig. 1.

Our data thus indicate that the *c-myc* *onc* gene is amplified in the genome of HL-60 cell line, as well as in the original uncultured leukaemic leukocytes which were obtained from the peripheral blood of the patient before any chemotherapeutic treatment¹⁸. The levels of *c-myc* amplification do not seem to vary during prolonged cell culture or after induction to differentiation. This result cannot be simply explained by amplification of specific chromosome(s) in these cells because they are hypodiploid and no hyperdiploidy of individual chromosomes was observed¹⁸. However, different amplified genes have been reported to be located in double minutes or homogeneous staining regions of chromosomes (for review, see ref. 19). Since double minutes have been described in the HL-60 cell line¹⁸, we are currently investigating whether these structures could contain amplified *c-myc* sequences. The *c-myc*-containing amplification unit must be larger than the 12.8 kb *Eco*RI fragment which is conserved in HL-60 DNA. The amplified fragment could be considerably larger, however, as amplified units ranging from 500 to 1,350 kb have been reported¹⁹. The amplified fragment does not contain any of the putative pseudogenes, indirectly suggesting that these are not in the immediate proximity of the *c-myc* gene in the human genome.

Although *c-myc* amplification is confirmed in HL-60 cells, we do not yet know whether *c-myc* is amplified in the other non-leukaemic cells of the same individual because tissues other than the leukaemic cells are not available from the patient. In the absence of these critical data, at least three different hypotheses can be formulated.

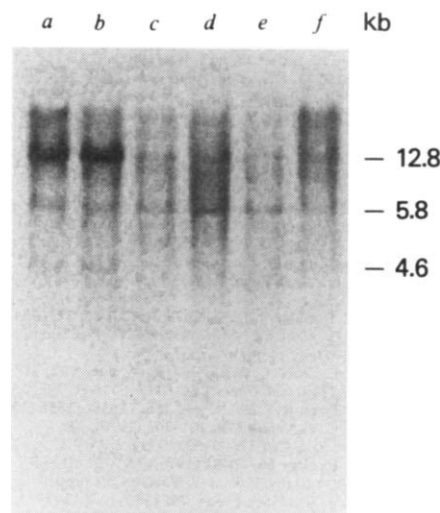


Fig. 2 Hybridization of HL-60 and other human DNAs to *v-myc* probe (pMCO). Thirty µg of DNA from: a, HL-60 passage 70; b, uncultured leukaemic HL-60 cells; c, normal human spleen; d, peripheral blood AML cells; e, Molt 4 cell line; f, peripheral blood normal human lymphocytes; were digested with *Eco*RI and hybridized to a *v-myc* probe (pMCO)¹⁰ as described in Fig. 1 legend.

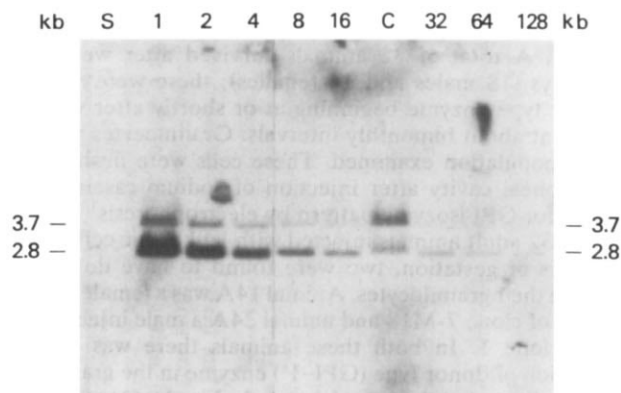


Fig. 3 Estimate of *c-myc* copy number in HL-60 DNA. For this Southern blot dilution experiment, all the DNAs listed below were digested with *Sst*I and simultaneously hybridized to pMC41-3RC and to a human *c-sis* probe (pL335)^{17,27}. *c-myc* and *c-sis* hybridization bands in 30 µg of DNA from normal human lymphocytes (lane C) were used as standards and their intensity compared with that obtained with the same amount of DNA from uncultured HL-60 cells (lane 1) and serial dilutions of the latter with equal amounts of *Sst*I-digested salmon sperm DNA. The reciprocal of the dilution factor is shown above the lanes. Salmon sperm DNA was chosen as diluting DNA as *c-myc*- or *c-sis*-related sequences are not detectable in these conditions of hybridization, as shown in lane S (30 µg). The 2.8 kb *c-myc* band in lane C is intermediate in intensity between the bands in lanes 16 and 32. The 3.7 kb band closely matches the one in undiluted NL-60 DNA. Therefore, the *c-myc* copy number is between 16- and 32-fold higher in HL-60 compared with the normal standard. In contrast, the unamplified *c-sis* gene is present as a single copy in both genomes. The slight difference in migration of diluted samples is due to decreased salt concentration after the dilution procedure.

(1) *c-myc* amplification has occurred in the germ line. As individuals of the same species usually have the same number of copies per cell of a given gene, germ-line amplification in a single individual would be novel and of, as yet, unknown significance. Another example of germ-line amplification also involves a cellular *onc* gene, *c-ras*, which is amplified in different but closely related murine species²⁰. Although this case differs from *c-myc* amplification in HL-60 as it affects an entire species²⁰, taken together, these preliminary observations could point to a relatively high genetic mobility of cellular *onc* genes.

(2) *c-myc* amplification may be present in other non-malignant myeloid cells, perhaps representing a normal event during myeloid differentiation analogous to the developmental amplifications of chorion genes during oogenesis in *Drosophila*²¹ or actin genes during myogenesis in chickens²². In this case, the 'freezing' of HL-60 cells by the transformation event in a stage when *c-myc* is amplified and thus expressed at high levels could correlate with the unique ability of HL-60 to propagate as a cell line in the absence of growth-stimulating factor(s)¹¹.

(3) *c-myc* amplification only in the leukaemic clone may have important implications for the pathogenesis of leukaemia in this patient. It has been proposed that increased expression of cellular *onc* genes may cause neoplastic transformation. Thus, the finding of *onc* gene amplification correlating with increased levels of expression in HL-60 cells suggests an alternative mechanism to the 'downstream' promotion of *onc* genes by viral promoters^{3-6,28}. The *onc* gene amplification model cannot be generally applied, at least in the case of *c-myc* and haematopoietic neoplasias, because this gene is neither amplified nor expressed⁸ above basal levels in a small group of fresh leukaemic cells or cell lines tested in this study. In particular, it is not amplified in three cases of APL tested, although no data are available on its expression in these cells. Alternatively, HL-60 cells may belong to a separate subgroup of APLs as they do not display the karyotypic abnormality involving a reciprocal translocation between chromosomes 15 and 17 (ref. 18) commonly found in this type of leukaemia²³. These observations suggest that if *c-myc* amplification is involved in leukaemic transformation in HL-60, different mechanisms, including activation of different cellular *onc* genes, may lead to transforma-

tion even in similar neoplastic diseases. The validity of the *onc* gene amplification model can be tested in several different tumours expressing high levels of any of the known cellular *onc* genes.

We thank T. S. Papas for a gift of the MC29 clone from which the pMCO probe was derived, E. P. Gelmann for preparing the pMCO recombinant plasmid, J. D. Rowley for providing leukaemic cells from some of the APL cases and Anna Mazzuca for secretarial assistance. R.D.F. is a Special Fellow of the Leukemia Society of America.

Note added in proof: Using a chicken *c-myc* probe Collins and Groudine have independently achieved similar results on *c-myc* amplification in cultured HL-60 cells (S. Collins, personal communication).

Received 28 May; accepted 17 June 1982.

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Participation of myeloid leukaemic cells injected into embryos in haematopoietic differentiation in adult mice

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It is of interest to determine to what extent malignant cells are still subject to control mechanisms that govern the growth and differentiation of their normal counterparts¹⁻⁴. One approach has been the introduction of malignant cells into a normal embryo. Such studies have shown that mouse teratocarcinoma cells injected into blastocysts can participate in normal development to produce adult chimaeric animals with markers derived from teratocarcinoma cells^{1-3,5-7}. Other types of malignant cells may also be able to participate in normal morphogenesis, provided that they have the ability to differentiate and are introduced into the embryo at an appropriate stage of development. Here we have examined myeloid leukaemic cells and our results indicate that injection of these cells into embryos *in utero* at 10 days of gestation can produce apparently healthy adult mice whose granulocytes contain a marker derived from the leukaemic cells.

Table 1 Microinjection of myeloid leukaemic cells into the fetal placenta *in utero*

Survival of injected animals	Control	Experimental injected on:	
		Day 10	Day 11
Number of females operated on	13	26	58
Females dead post-operation	3	5	9
Females aborting	6	11	28
Females pregnant at term	4	10	21
Number of fetuses injected	141	268	469
Fetuses in females pregnant at term	33	94	156
Viable progeny	16	39	90
Animals dead after birth	2	7	35
Animals surviving	14	32	55

Fetuses were microinjected through the uterine wall into the fetal placenta¹². Each injection contained $\sim 5 \times 10^4$ cells and the cells were injected in the culture medium used for growing the leukaemic cells¹¹ without serum. Of 129 animals born, 70 were injected with leukaemic clone 7-M18 and 59 with leukaemic clone 1. Of 87 surviving animals, 45 were injected *in utero* with clone 7-M18 and 42 with clone 1. There was no statistically significant difference between groups receiving either of these clones and these data have therefore been pooled. Controls were injected with culture medium alone. Of the 84 females injected with leukaemic cells in these experiments, 75 were C3HeB and 9 were C57BL/6. The two apparently healthy adult mice with donor type enzyme (Table 2) were both obtained after injection of leukaemic cells into the fetal placenta of C3HeB mice.

We have used in these studies two clones of mouse myeloid leukaemic cells that grow in culture as myeloblasts to promyelocytes and which differ in their capacity to be induced to differentiate to mature macrophages and granulocytes by the normal macrophage and granulocyte differentiation-inducing protein MGI-2^{4,8-11}. One clone (7-M18) could be induced by MGI-2 to differentiate in culture to mature macrophages or granulocytes (mainly macrophages), whereas the other clone (1) could not be induced by MGI-2 to differentiate in culture to mature cells^{10,11}. To test for the incorporation of a marker from the leukaemic cells into the haematopoietic cell system, fetal mice were injected *in utero* with the myeloid leukaemic cells at 10 or 11 days of gestation. The leukaemic cells were from the strain SJL and the host animals injected were strains C3HeB or C57BL/6. Both clones of leukaemic cells contain the slow migrating glucose phosphate isomerase (GPI) isozyme GPI-1^a, whereas cells from C3H and C57BL/6 mice are homozygous for the fast migrating isozyme GPI-1^b. Leukaemic cells were microinjected into the placenta of each fetus according to the procedure described for injection of normal haematopoietic cells¹² and the numbers of viable progeny obtained are shown in Table 1.

In the first series of experiments, leukaemic cells were injected on day 10 of gestation (the day of plug was designated day 0) and 39 animals were born. One litter of four animals was cannibalized by the mother shortly after birth and another

three animals died, were partially eaten and could not be examined. A total of 32 animals survived after weaning at 21–28 days (18 males and 14 females); these were screened for donor type enzyme beginning at or shortly after weaning, and then at about bimonthly intervals. Granulocytes were the first cell population examined. These cells were flushed from the peritoneal cavity after injection of sodium caseinate and analysed for GPI isozyme pattern by electrophoresis¹³.

Of the 32 adult animals injected with leukaemic cells *in vivo* at 10 days of gestation, two were found to have donor type enzyme in their granulocytes. Animal 14A was a female injected with cells of clone 7-M18 and animal 24A a male injected with cells of clone 1. In both these animals there was a major contribution of donor type (GPI-1^a) enzyme in the granulocyte samples collected at the age of 1 month (Fig. 1). Densitometric analysis of the electrophoretic pattern indicated that in these samples the donor type enzyme contributed at least 25% of the GPI activity. In both animals this contribution gradually decreased with age. We found 15–20% donor-derived GPI activity in the granulocytes at 3 months of age, 10% at 5 months, and only 5% at 7 months. This isozyme was no longer detected at 8.5 months in animal 14A and 10 months in animal 24A. A minor contribution of donor type isozyme was also detected in the leukocytes isolated from the peripheral blood up to 3 months of age in the case of animal 14A and 5 months in the case of animal 24A and this could be accounted for by the granulocytes in the peripheral blood. However, this isozyme was not detected in the peritoneal macrophages in these mice (Table 2). The leukaemic cells injected into the fetuses could be readily distinguished from normal peritoneal granulocytes (Fig. 2). At least 1,000 cells per sample were examined microscopically, and no leukaemic cells were seen in the peritoneal exudate or peripheral blood cells in these or any of the other adult mice. At autopsy, when the granulocytes no longer showed donor type isozyme, there was also no detectable amount of this enzyme in the spleen, thymus, bone marrow, kidney, liver or brain.

In a second series of experiments, leukaemic cells were injected at day 11 instead of at day 10 of gestation. In this series of injections 90 viable progeny were obtained, of which only 55 survived beyond weaning. None of these 55 mice showed detectable donor type GPI in their peritoneal granulocytes or macrophages. In these experiments 39% of the injected animals died before weaning. In some cases the carcasses had been partially eaten or post mortem changes were too marked to

Table 2 Donor type GPI isozyme in adult animals microinjected *in utero* with myeloid leukaemia

Animal no.	Clone injected	Age tested (months)	% Donor-type isozyme in:			
			Peritoneal cells		Peripheral blood	
			Granulocytes	Macrophages	Leukocytes	Erythrocytes
14A	Clone 7-M18	1	25	0	3	0
		3	20	0	3	0
		5	10	ND	0	0
		7	5	ND	0	0
		8.5	0	0	0	0
24A	Clone 1	1	25	0	3	0
		3	15	0	3	0
		5	10	ND	3	0
		7	5	ND	0	0
		10	0	0	0	0

Peritoneal granulocytes and macrophages were collected at 16 and 72 h, respectively, after intraperitoneal injection of 10% sodium caseinate solution. The peritoneal cell populations contained on average $\sim 90\%$ granulocytes and 80% macrophages. The other cells in the peritoneal samples used were lymphocytes and no leukaemic cells were observed. Peripheral blood samples were collected from the retro-orbital sinus and the erythrocytes and leukocytes separated. Leukaemic cells were also not observed in these samples. Cell pellets were suspended in water, lysed by three cycles of freezing and thawing, and the supernatants, obtained after centrifugation for 30 min at $28,000g$, were electrophoresed on starch gels and stained as described elsewhere¹³. Per cent donor-type enzyme values represent estimates based on densitometric analyses of the electrophoretic pattern of GPI. ND, not determined.

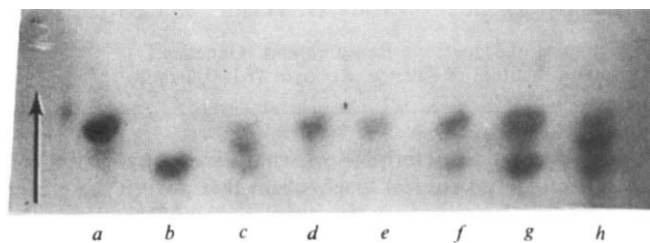


Fig. 1 Glucose phosphate isomerase isozyme analysis by starch gel electrophoresis of extracts from peritoneal exudate cells. Granulocytes or macrophages were collected from the peritoneal cavity after injection of sodium caseinate. No leukaemic cells were observed. *a*, C3H control granulocytes; *b*, SJL control granulocytes; *c*, F1 (C3H x SJL) heterozygous control macrophages; *d*, mouse 30A, granulocytes at 4 weeks after birth (no donor-type enzyme); *e*, mouse 32A, granulocytes at 4 weeks after birth (no donor-type enzyme); *f*, mouse 14A, granulocytes at 3 months after birth (host and donor-type enzymes present); *g*, mouse 24A, granulocytes at 4 weeks after birth (host and donor-type enzymes present); *h*, F1 (C3H x SJL) heterozygous control granulocytes.

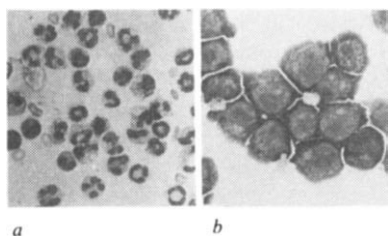


Fig. 2 *a*, Peritoneal granulocyte population collected from healthy adult mice; *b*, myeloid leukaemic cells (clone 7-M18). Cells stained with May-Grunwald-Giemsa. $\times 630$.

allow enzymatic examination. Of the remaining 18 mice that died before weaning, 9 had tumours derived from the injected leukaemic cells, as determined by GPI analysis; the rest appeared to predominantly have severe bacterial pneumonia. The animals died with tumours by 15 ± 1 days after birth, that is, 3.5 weeks after injection of the leukaemic cells. The mean survival time of the tumour-bearing animals injected *in utero* was similar to that of neonatal syngeneic mice injected within 12 h of birth with an equivalent number (5×10^4) of leukaemic cells.

The present experiments indicate that malignant cells other than teratocarcinoma cells injected into embryos may participate in normal development. In two animals injected *in utero* with myeloid leukaemic cells on day 10 of gestation, at least 25% of the glucose phosphate isomerase in the peritoneal granulocytes was donor specific at 1 month after birth. Leukaemic cells were not seen in the cell populations analysed and these adult mice seemed healthy. These results indicate that the myeloid leukaemic cells participated in haematopoietic differentiation in apparently normal adult mice. The donor type enzyme decreased gradually over a period of 7 months and was no longer detectable at the age of 8–10 months. The selection of one cell type over another has also been reported in haematopoietic chimaeric animals obtained after injection of normal haematopoietic cells¹², and in allophenic mice produced from aggregated blastomeres^{14,15}. Cells with a donor-derived marker may have a selective disadvantage compared with the host cells, resulting in a gradual decrease in their contribution to the total cell population, perhaps due to the presence of a limited number of precursors.

Injection of leukaemic cells into 10-day-old fetuses resulted in two adult mice having cells with donor type isozyme whereas injection into 11-day-old fetuses did not and gave some animals with tumours. Further experiments with larger number of animals are required to determine the importance of a specific stage of embryonic development for obtaining adult animals with a donor type marker. We injected the embryos at the stage when there appears to be considerable migration of the normal progenitors of granulocytes and macrophages¹⁶. Other tumour cells may need to be injected into blastocysts¹⁷. This has given chimaeras with teratocarcinoma^{1-3,5-7} but not with melanoma cells¹⁸. Others may need to be injected at different stages of development into the placenta¹², or directly into the fetus¹⁹. When leukaemic cells are induced to differentiate *in vitro* by the normal differentiation-inducing protein MGI-2, it is the leukaemic cells and not segregants that differentiate to mature cells^{4,9}. It has also been shown that leukaemic cells may differentiate better *in vivo* than *in vitro*²⁰. The finding of a donor cell marker in animals injected as embryos with malignant cells suggests that the malignant cells may respond to the controls that regulate normal growth and differentiation in the embryo, or the cells with a donor cell marker may be segregants with a non-malignant phenotype derived from the malignant cells, as can occur with sarcomas⁸. The use of additional donor-specific markers including chromosome markers should make it possible to distinguish between these possibilities.

We thank Professor H. B. Stein for his assistance in histopathological examination and Professor H. R. Lindner for his

helpful comments. This work was supported in part by a grant to H. R. Lindner from the Ford Foundation and the Rockefeller Foundation, New York, and to C.G.W. from the Binational Science Foundation. C.G.W. is the incumbent of the Pauline Recanati Career Development Chair in Immunology.

Received 8 March; accepted 25 June 1982.

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Production of a monoclonal antibody specific for Hodgkin and Sternberg-Reed cells of Hodgkin's disease and a subset of normal lymphoid cells

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The origins of the neoplastic cells in Hodgkin's lymphoma¹, the Hodgkin (H) and Sternberg-Reed (SR) cells, are still obscure, and these cells appear to carry no markers found on any population of normal cells^{2,3}. However the recent establishment of permanent Hodgkin cell lines^{3,4} has led to the search for tumour-specific antigens and/or for membrane markers on H and SR cells which may indicate the normal equivalent cells. Here, we describe the production of mouse monoclonal antibodies against the Hodgkin cell line L428. One hybridoma antibody, Ki-1, was found to be specific for H and SR cells of all Hodgkin's lymphomas tested and a minute, but distinct new cell population in normal tonsils and lymph nodes.

The existence of H and SR cell specific antigens was suggested by a conventional rabbit antiserum raised against the L428 cell line. After absorption with various normal human cells, the anti-L428 antiserum proved to be selectively reactive with L428 cells and H and SR cells⁵. To further investigate these apparent Hodgkin-specific tumour antigens we have started to produce hybridoma antibodies directed against H and SR cell antigens⁶.

BALB/c mice were immunized with L428 cells according to the schedule described by Stähli *et al.*⁷. Spleen cells were fused with the non-secreting myeloma line X63-Ag8.653 (ref. 8). In one fusion experiment we selected 57 pre-cloned 'resp.' highly enriched hybridomas that showed strong reactivity with L428 cells and no reactivity with a 10 times higher number of pooled normal tonsil cells. The resulting 57 monoclonal antibodies were tested by using the immunoperoxidase staining technique on frozen sections of biopsy tissue infiltrated with Hodgkin's

disease^{9,10}. One of the hybridoma antibodies reacted with H and SR cells, but not with other cells in the biopsy tissue. This clone was designated Ki-1. The constant expression of the Ki-1 antigen on H and SR cells was confirmed by immunostaining tissue from 14 cases of Hodgkin's disease of the four main histological types (Table 1). The H and SR cells of all cases stained distinctly (Fig. 1), but the intensity of the staining varied. There was no correlation between intensity of staining and histological type.

The specificity of the Ki-1 antibody for H and SR cells was further demonstrated, as the antibody did not stain cells of more than 50 cases of non-Hodgkin's lymphomas of various types, one case of osteomyeloclerosis, two cases of malignant histiocytosis and two cases of histiocytosis X. The Ki-1 antibody also did not react with cells of normal peripheral blood, skin, liver, kidney, lung or brain, or with macrophages of different types or stages of differentiation (Table 2).

Stainings with a highly sensitive three-layer immunoperoxidase labelling technique of a large series of normal or hyperplastic tonsils and lymph nodes, however, yielded unexpected results. A small population of cells located around and between cortical follicles and, to a lesser extent, at the inner rim of follicular mantles showed weak, but distinct staining for Ki-1 (Fig. 2). These cells were smaller than typical H and SR cells and had one nucleus; the nucleolus was prominent, like that of H and SR cells. Such cells could not be demonstrated with the conventional rabbit antiserum raised against L428 cells that reacted specifically with H and SR cells. There are two possible explanations for this phenomenon: (1) the conventional rabbit anti-L428 serum recognizes an antigen different from

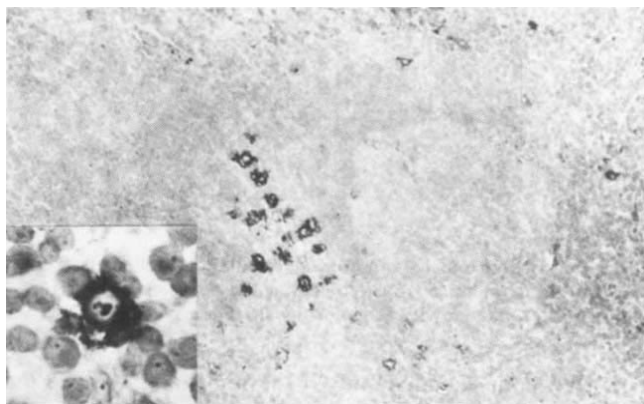


Fig. 2 A cluster of Ki-1-positive cells in a hyperplastic (non-malignant) human tonsil. They are chiefly located between, around and sometimes within the B-cell follicles. In other areas of the tonsil, there are fewer Ki-1 positive cells. Inset: Ki-1-positive cell at high power view. Note the sharply defined prominent nucleolus within the pale nucleus and the labelled abundant cytoplasm.

that detected by monoclonal antibody Ki-1, or (2) both reagents detect the same antigen, which is strongly expressed on H and SR cells but only weakly on the normal Ki-1-positive cells. The second possibility is conceivable, because the multiple-step absorbed rabbit anti-L428 serum had to be used at rather high concentrations even for the detection of H and SR cells. Thus, the titre might have been too low to produce sufficient staining of normal cells expressing only low amounts of the antigen.

We also investigated normal human bone marrow as a possible source of various precursor cells of the peripheral lymphoid tissues and blood. Stainings with a three-layer immunoalkaline phosphatase technique revealed only very few scattered Ki-1-positive cells among the haematopoietic cells. From the non-reactivity of Ki-1 antibody in a case of osteomyeloclerosis (Table 2), which is a neoplastic proliferation of cells of the granulopoiesis, erythropoiesis and thrombopoiesis, we conclude that Ki-1-positive cells in the normal bone marrow do not belong to one of those three major myeloid cell lineages.

The detection of Ki-1 antigen on a small number of normal cells in tonsils, lymph nodes and bone marrow suggests that it may be a differentiation antigen and not a virus-induced or tumour-specific antigen. The diagnostic value of the Ki-1 monoclonal antibody is not reduced by the occurrence of Ki-1 antigen on normal cells, because these cells are easily distinguishable from H and SR cells by morphological criteria. The expression of Ki-1 antigen on H and SR cells of all cases of Hodgkin's disease studied and the absence of Ki-1 antigen in non-Hodgkin's lymphomas demonstrates that this antigen may be used as a reliable marker for Hodgkin's disease. The constant expression of Ki-1 antigen on H and SR cells in all histological types of Hodgkin's disease also supports the view that H and SR cells of all variants of Hodgkin's disease are homogeneous in origin and nature and not heterogeneous as assumed by some investigators¹¹⁻¹⁴.

Table 1 Reactivity of cells of Hodgkin's disease with the antibody Ki-1

Histological type	No. of cases	No. of cases showing H and SR cells reactive with:		
		SK203 (directed at HLA-DR)	W6/32HK (inactive control)	Ki-1
Lymphocyte predominance	2	1	0	2
Nodular sclerosis	5	3	0	5
Mixed cellularity	5	4	0	5
Lymphocyte depletion	2	1	0	2
Total	14	9	0	14

Fig. 1 Hodgkin and Sternberg-Reed cells in tissue infiltrated by Hodgkin's disease reacting selectively with the monoclonal antibody Ki-1. Lyophilized 8 µm sections of frozen tissue biopsies were fixed in acetone for 10 min and subsequently in chloroform for 10 min, both at room temperature. The fixed sections were then incubated with undiluted hybridoma supernatants or hybridoma ascitic fluids (dilutions ranged between 1:1,000 and 1:50,000) for 30 min. After a short wash in phosphate-buffered saline (PBS) the sections were treated with peroxidase-conjugated anti-mouse Ig (Dako) for 30 min, followed by a short wash. This reagent was reabsorbed with insolubilized human serum and diluted 1:10 in PBS to which normal human serum (final dilution of 1:5) was added to block cross-reactivity against human immunoglobulin. To increase sensitivity, stainings were performed at 37 °C or the sections were incubated with a third antibody layer composed of peroxidase-conjugated goat anti-rabbit IgG antibody (Medac) diluted 1:10 in PBS to which normal human serum (final dilution of 1:10) was added. After a brief wash, peroxidase was made visible by incubating the sections with diaminobenzidine (0.6 mg per ml) and hydrogen peroxide (0.01%) for 10 min at room temperature. The sections were then washed in PBS, counterstained with haemalum and mounted for microscopic examination. Negative controls were performed with the non-reactive monoclonal antibody W6/32HK²⁵ and positive controls with a set of monoclonal antibodies reactive with IgM, IgD (Bethesda), T-cells (OKT3, Ortho) HLA-DR (Becton Dickinson) and other cells or antigens.

Table 2 Reactivity of normal and malignant cells of various tissues with the antibody Ki-1

	No. of samples	Cells reactive with:		
		SK203 (directed at HLA-DR)	W6/32HK (inactive control)	Ki-1
Lymph nodes and tonsils	10	B-cells, macrophages, interdigitating cells	None	Usually only few, but sometimes clusters of cells showing weak reaction around, between and at inner rim of follicular mantles
Blood	5	Monocytes, B-cells	None	None
Bone marrow	2	Various cells	None	Very few, scattered cells
Thymus	1	Most of the non-lymphoid framework cells	None	Very few cells in the medulla
Lung	2	Macrophages	None	None
Kidney	3	Endothelial cells, intertubular fibrocytes, proximal tubules (weak reaction)	None	None
Liver	2	Kupffer's cells	None	None
Brain	1	Not done	None	None
Skin	2	Langerhans' cells	None	None
Piringer's lymphadenitis	1	B-cells, macrophages, interdigitating cells	None	Interfollicular areas with relatively many labelled cells
B-type chronic lymphocytic leukaemia	10	Tumour cells of all cases	None	None
Follicular lymphoma	15	Tumour cells of all cases	None	None
Lymphoblastic lymphoma of Burkitt type	5	Tumour cells of all cases	None	None
B-type large-cell lymphoma	10	Tumour cells of 4 of 5 cases	None	None
T-type chronic lymphocytic leukaemia	1	None	None	None
Lymphoblastic lymphoma of thymocytic type	3	None	None	None
Various lymphomas of peripheral T-cell type	8	None	None	None
Osteomyelosclerosis (containing nearly all maturation forms of the granulocyte- and thrombopoiesis)	1	Not done	None	None

So far, the cells from which H and SR cells are derived have not been identified. The small subset of cells detected by the Ki-1 antibody in normal lymphoid tissue may possibly give rise to H and SR cells in appropriate conditions. This may be the case as Hodgkin's disease usually starts to develop in those areas in which most of the small Ki-1-reactive normal cells are found, namely, the parafollicular area¹⁵. The Ki-1-positive normal cells were investigated using double immunofluorescent labelling and three-layer immunoperoxidase labelling of serial sections of tonsils and a case of Piringer's lymphadenitis, containing clusters of Ki-1-positive cells. Only the latter method was sensitive enough to obtain clear-cut results. The comparison of the single stained serial sections showed that the normal cell population expressing Ki-1 antigen does not react with antibodies directed at B cells (anti-IgM, anti-IgD), T cells (OKT11, ref. 16), macrophages (OKM1, ref. 17, monocyte.1, ref. 18 and monocyte 2, G. Numez, *et al.*, in preparation and lysozyme), dendritic reticulum cells (R4/23, ref. 19), interdigitating reticulum cells (T-ALL 2, ref. 20), or cells of the granulopoiesis (Tü5, Tü6, Tü9, ref. 21, 3C4, ref. 22), erythropoiesis (anti-glycophorin A, ref. 23) or thrombopoiesis (J-15, AN-51, ref. 24). Thus, these data indicate that the Ki-1-positive normal cells lack markers specific to, or characteristic of all known cell types of the haemato-lymphoid system. It may be that the Ki-1 antibody detects a new, so far unidentified cell population in normal lymphoid tissue.

We thank Dr A. McMichael for the monoclonal antibodies J-15 and AN-51, Dr P. A. W. Edwards for monoclonal antibody against glycophorin A, and Dr A. Ziegler for monoclonal antibody W6/32HK. The technical assistance of Heinke Asbahr, Ilse Horn, Angela Gelhaus and Kirsten Tiemann is acknowledged. This work was supported by Deutsche Krebshilfe e.V., Schleswig-Holsteinische Krebsgesellschaft e.V. and Deutsche Forschungsgemeinschaft SFB 111, projects D7 and D8.

Received 29 March, accepted 24 June 1982.

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HLA-restricted lymphoproliferative responses to MN blood group determinants

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It is well established that strong proliferative responses in mixed lymphocyte culture (MLC)¹ are controlled by gene products of the *HLA-D* locus, but stimulation between *HLA-D* identical individuals does occur and may partly be due to differences at the *HLA-A*, or *HLA-B* loci. However, many unrelated individuals who type identically for *HLA-A*, *-B*, *-C*, and *-D/DR* determinants have also been shown to be mutually stimulatory. The development of the primed lymphocyte test (PLT) provided a powerful tool in the identification of these non-*HLA* loci²⁻⁹. Using this test, we show here that significant *HLA*-restricted proliferative responses occurred in association with M or N blood group antigens.

Lymphocytes, separated from freshly drawn blood, were primed to stimulating cells from allogeneic donors in long-term MLC as described previously⁹. Lymphocytes were typed for HLA-A, -B, and -C antigens by the microdroplet lymphocytotoxicity test with a battery of highly selected typing antisera¹⁰. HLA-DR typing was performed on a B-cell enriched lymphocyte population¹⁰. HLA specificities were defined on the basis of reactivity with sera from the VIIth and VIIIth International Histocompatibility Workshops.

To investigate the role of the M and N determinants in PLT, lymphocytes from M-negative or N-negative donors were primed in long-term cultures against cells from donors mismatched for the corresponding M or N antigen. These responder-stimulator combinations were also mismatched for one or two HLA-DR antigens. Primed lymphocytes were then tested for proliferative responses against stimulating cells from third-party allogeneic donors. Data from one experiment demonstrating the role of the M determinant in PLT are given in Table 1. Lymphocytes from donor A (HLA-A2, A26; Bw16, Bw44; DRw8; Nss) were primed against cells from donor B (HLA-A1, Aw24; B8, Bw58; Cw6; DR1; MNs) in long-term MLC. Primed lymphocytes were tested for proliferative responses against stimulating cells from 10 allogeneic donors. Primed lymphocytes responded to three donors (C, D, E) who are positive for the sensitizing DR1 specificity. Primed lymphocytes also responded to three M-positive, HLA-A2-positive, HLA-DR1-negative donors (F, G, H). On the other hand, primed lymphocytes did not respond to two M-positive A2-negative, DR1-negative donors (I, J) and to two M-negative, DR1-negative donors (K, L). Similar data were observed in two other PLT experiments; (1) HLA-A3, A29; B8, Bw45; Cw6; Ns anti-A2, A28; Bw39, Bw50; DR2, DRw6; MS, and (2) HLA-A1, B7, B8; DR3, DR5; Ns anti-A2, A28; Bw39, Bw50; DR2; MS. The data from these three experiments are summarized in Fig. 1a. Primed cells responded to cells from donors who are positive for the sensitizing DR specificity. These primed lymphocytes also responded to lymphocytes from M-positive, sensitizing DR-negative individuals who shared HLA-A antigen(s) with the responder cells. However, primed lymphocytes did not respond to lymphocytes from M-positive, specific-DR-negative donors who did not share HLA-A determinant(s) with the responder cells. Primed lymphocytes did not respond to stimulating cells from M-negative, specific-DR-negative donors (data not shown).

Data from an experiment demonstrating the role of the N determinant in PLT are given in Table 2. Lymphocytes from a donor A (HLA-A2, A28; Bw39, Bw50, DR2, DRw6; MS) were primed against cells from donor B (HLA-A1; B7, B8; DR3; DR5; Ns). Primed lymphocytes were tested for proliferative responses against stimulating cells from 12 allogeneic donors. Primed lymphocytes responded to four donors (C, D, E, F) who are positive for the sensitizing DR3 or DR5

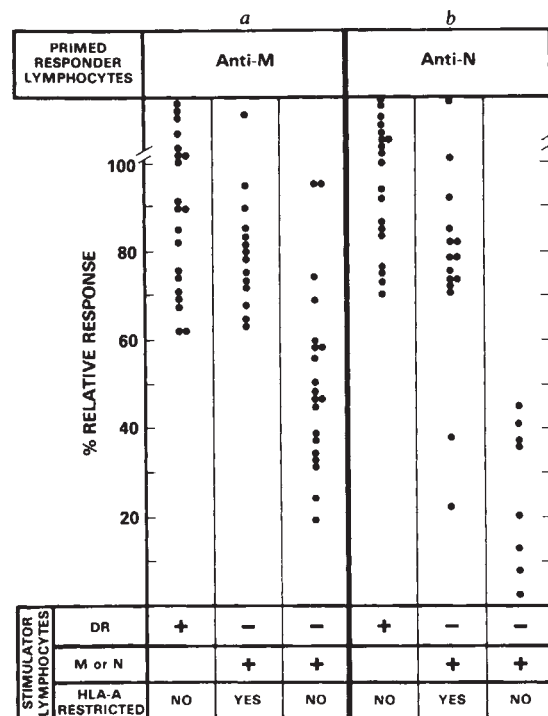


Fig. 1 Responses of primed lymphocytes to allogeneic lymphocytes in the primed lymphocyte test. *a* Shows the responses of primed lymphocytes to the sensitizing HLA-DR and M determinants (data pooled from three separate experiments); *b* shows the responses of primed lymphocytes to the sensitizing HLA-DR and N determinants (data pooled from three separate experiments). A PLT response to allogeneic donor cells was considered positive when the per cent relative response (% RR) was more than the lower limit of the mean ± 2 s.d. (95% confidence limits) of the PLT responses to the DR-specific allogeneic donors. Cut-off point for the anti-M PLT response is 61%; for anti-N 67%.

specificity. Primed lymphocytes also responded to three N-positive, HLA-A2-positive, HLA-DR3, 5-negative donors (G, H, I), but did not respond to three N-positive, A2-negative, DR3,5-negative donors (J, K, L) or to two N-negative, DR3,5-negative donors (M, N). No response to the s determinant was observed in this experiment. Similar data were observed in two other PLT experiments; (1) HLA-A2, A28; Bw39, Bw50; DR2, DRw6; MS anti-A3, A29; B8, Bw45; Cw6; DR3; Ns, and (2) HLA-A1, A3; Bw35, Bw52; Cw4; DR1; Ms anti-A2, A28; B14, Bw60; CW3; DR7; MNs (Fig. 1b).

Highly significant associations were found between PLT and

Table 1 Responses of primed lymphocytes to M determinant in PLT

Stimulating cell	HLA type -A, -B, -C	-DR	MNSs	% Relative response	PLT determinant
B	A1, Aw24; B8, Bw58; Cw6	1	MNs	100	+
C	Aw24, Aw33; B14, Bw63	1	MSs	145	+
D	A11, Aw33; B14, Bw44; Cw1	1	MNs	176	+
E	A1, Aw23; Bw44, Bw57; Cw6	1	MNSs	103	+
F	A1, A2; B8, Bw62; Cw3	3	MNSs	100	+
G	A1, A2; Bw39, Bw58; Cw6	7, w8	MSs	102	+
H	A2, Aw24; B7, Bw62; Cw3	4, w8	MSs	99	+
I	A1, Aw23; Bw44, Bw51	3, w6	MSs	58	-
J	A11; Bw62; Cw1, Cw3	3	MNs	34	-
K	A1; B7, B8	3, 5	Ns	16	-
L	A2, A3; B7, B8; Cw1	2	Ns	58	-

Lymphocytes from donor A (HLA-A2, A26; Bw16, Bw44; DRw8; Nss) were primed against cells from donor B in MLC. Primed lymphocytes were used at a concentration of 25×10^5 cells per well.

Table 2 Responses of primed lymphocytes to N determinant in PLT

Stimulating cell	HLA type -A, -B, -C	-DR	MNSs	% Relative response	PLT determinant
B	A1; B7, B8	3,5	Ns	100	+
C	A1, A2; B8, Bw62; Cw3	3	MNSs	109	+
D	A3, A29; B8, Bw45; Cw6	3	Ns	94	+
E	A11, Aw30; B13, B18; Cw6	5, 7	MNS	86	+
F	A2, A29; B7, Bw60	4, 5	MSs	73	+
G	A2, A3; B7, B27; Cw1	2	Ns	92	+
H	A2, A26; Bw16; Bw44	w8	NSs	73	+
I	A2, A28; B14, Bw60; Cw3	7	MNS	82	+
J	A11, Aw33; B14, Bw44; Cw1	1	MNS	45	-
K	A1, A11; Bw35, Bw37; Cw4	1	MNSs	37	-
L	A1, Aw23; Bw44, Bw57; Cw6	1	MNSs	36	-
M	Aw24, Aw33; B14, Bw63	1	MSs	22	-
N	A1, A3, Bw35, Bw52; Cw4	2	Ms	50	-

Lymphocytes from donor A (HLA-A2, A28; Bw39, Bw50; DR2, DRw6; MS) were primed against cells from donor B in MLC. Primed lymphocytes were used at a concentration of 25×10^3 cells per well.

HLA-DR determinants, and between PLT and HLA-restricted M or N determinants (Table 3). No differences were observed in the responses of responder lymphocytes to stimulator cells from homozygous or heterozygous M or N individuals. In our panel of normal healthy subjects, we have only one HLA-D/DR-identical, M- or N-mismatched pair. Primed lymphocytes (HLA-A1; B7, B8; DR3, DR5, Ns anti-A1, A3; B8, Bw35; DR3, DR5; MNS) responded to four M-positive donors who shared an HLA-A antigen with the responder lymphocytes. Primed lymphocytes did not respond to M-positive stimulators who did not share an HLA-A antigen.

To investigate the role of the S and s genes in PLT, lymphocytes were primed in responder-stimulator combinations mismatched for the S (or s) and for one or two HLA-DR antigens. Primed lymphocytes were tested for proliferative responses against stimulating cells from third-party allogeneic donors. Lymphocytes from third-party donors, who are positive for the sensitizing DR antigen, elicited strong proliferative response in PLT. However, primed lymphocytes did not respond to stimulating cells from donors who are positive for the specific S or s determinant but negative for the sensitizing DR specificity. These third-party donors included individuals who shared HLA-A determinants(s) with the responder PLT

cells. This was observed in six experiments using different responder-stimulator combinations. Thus, no effects of S or s incompatibility were observed in PLT.

The above findings demonstrate that lymphocytes from individuals mismatched for M and N determinants can cause significant HLA-restricted proliferative responses in PLT. It appears that lymphoproliferative responses in the PLT to stimulating lymphocytes from M and N mismatched individuals require the simultaneous expression of the same HLA-A antigen(s) on both stimulator and the primed responder lymphocytes. On the other hand, no effects of S and s incompatibilities were observed in the PLT.

MNSs determinants appear to be associated with two human erythrocyte membrane sialoglycoproteins¹¹⁻¹³. The M and N determinants are associated with glycophorin A, while the S and s determinants are associated with glycophorin B¹⁴. Similar sialoglycoproteins have been demonstrated in the membranes of other cells such as platelets, granulocytes and lymphocytes^{14,15}. Whether these glycoproteins are associated with the M and N antigens in these cells is unclear.

Blood group incompatibility has been shown to play a part in organ transplantation in man. Graft versus host disease in patients receiving bone marrow transplants from HLA-identical siblings has recently been correlated with MNSs incompatibility¹⁶. The present data suggest that MN determinants, or closely related genes, are expressed on lymphocytes and can cause HLA-restricted proliferative responses in PLT which may be an important consideration in organ transplantation.

This work was supported by the MRC of Canada. We thank Dr W. Rawls of McMaster University and Dr M. Schanfield of the American Red Cross for helpful suggestions during the preparation of this manuscript.

Table 3 Association between HLA-A-restricted M or N determinants and HLA-DR in PLT

	Association*				χ^2	r	P
	+/+	+/-	-/+	-/-			
PLT/M(HLA-A restricted)	14	8	0	23	18.38	0.69	$<10^{-4}$
PLT/HLA-DR	20	22	0	23	13.66	0.49	$<10^{-3}$
PLT/N(HLA-A restricted)	13	7	2	19	11.30	0.58	$<10^{-3}$
PLT/HLA-DR	19	20	0	21	12.81	0.50	$<10^{-3}$

A PLT response was considered positive or negative using the criteria given in Fig. 1. The data were analysed using 2×2 comparisons of the determinants recognized by PLT and HLA-DR. For PLT/M or N(HLA-A restricted) comparisons, allogeneic donors positive for the sensitizing HLA-DR antigens were excluded from the analysis.

* Association terminology (for example, for PLT/M(HLA-A-restricted): +/+ indicates a positive PLT test associated with the presence of the M antigen and HLA-A restriction; +/- indicates a positive PLT test associated with the absence of the M antigen and HLA-A restriction; -/+ indicates a negative PLT test associated with the presence of the M antigen and HLA-A restriction; -/- indicates a negative PLT test associated with the absence of the M antigen and HLA-A restriction.

Received 6 January; accepted 15 June 1982.

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Monoclonal antibody defines a macrophage intracellular Ca^{2+} -binding protein which is phosphorylated by phagocytosis

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The macrophage is a highly phagocytic cell which has been widely used in attempts to dissect the mechanisms of endocytosis¹. In immune phagocytosis specific interactions occur between ligands on the presenting particle and Fc or C3b specific receptors expressed on the plasma membrane^{2,3}, but macrophages also avidly ingest particles such as polystyrene latex by an immunologically nonspecific mechanism. This process is guided by sequential ligand-receptor interactions^{4,5}. Biochemical studies indicate that actin, myosin and several regulatory components distinct from the troponin system of skeletal muscle, take part in phagocytosis by macrophages and polymorphonuclear leukocytes (PMN)⁶ and indirect immunofluorescence confirms the presence of these components at sites of phagocytic activity⁷. However, comparatively little is known about coupling between receptor binding and assembly of the contractile system, although electrical changes⁸ and the flux of divalent cations⁹ have been implicated. Here we report a monoclonal antibody that detects an intracellular protein from macrophages which binds Ca^{2+} in non-phagocytosing cells, and becomes phosphorylated as a result of phagocytosis. It might therefore take part in signal transduction during this process.

In an attempt to study antigenic components of the cytoplasmic face of the mouse macrophage phagolysosome membrane, rats were hyperimmunized with latex-containing phagolysosomes (LP) prepared by flotation in a discontinuous sucrose gradient¹⁰. Antibodies against LP were detected by trace indirect radioimmunoassay (RIA) and rats with high serum titres, >1/1000, used as spleen donors in myeloma fusions¹¹. Hybridoma supernatants containing antibodies for antigens on the cytoplasmic face of the phagolysosome membrane were screened on LP or on cells permeabilized by methanol fixation and positive cultures were cloned twice in soft agar. The present report studies PL4, one monoclonal antibody produced in this way.

The binding properties of PL4 were examined on intact or methanol-fixed thioglycollate-elicited mouse peritoneal macrophages (TPM), on LP isolated from such macrophages, and on mouse L-cell fibroblasts, which lack specific phagocytic pathways, but are able to ingest latex. The antigen it bound could be detected by RIA on LP (Fig. 1a), and in methanol-fixed, but not live TPM, even in the absence of a phagocytic stimulus (not shown). It was also detectable by RIA (Fig. 1b) and by FACS analysis (Fig. 1c) of permeabilized, but not intact (Fig. 1d) fibroblasts. Another monoclonal antibody, F4/80, which detects an external plasma membrane component of the macrophage¹², did not bind to isolated LP. Indirect immunofluorescence with fixed cells showed a granular cytoplasmic distribution, not confined to phagocytosing cells or the vicinity of phagolysosomes and the antibody recognizes a similar antigen in human and monkey, but not rat cells. We concluded that the antigen detected by PL4 is restricted to the inside of the cell and is present in macrophages as well as other cells, including cells which have not received a phagocytic stimulus.

If the inorganic phosphate pool within mouse macrophages is equilibrated with ^{32}P -labelled P_i then a number of proteins associated with isolated LP become phosphorylated. PL4 specifically precipitates a labelled protein from lysates prepared from TPM after phagocytosis of latex microspheres (Fig. 2a,

arrow). This protein has a molecular weight of ~19,000 as estimated by SDS-polyacrylamide gel electrophoresis and was not detected as a phosphorylated molecule in control preparations without specific antibody or in the absence of a phagocytic stimulus (Fig. 2b-d).

If protein blotting techniques are used to produce nitrocellulose replicas of SDS-polyacrylamide gels then it is possible

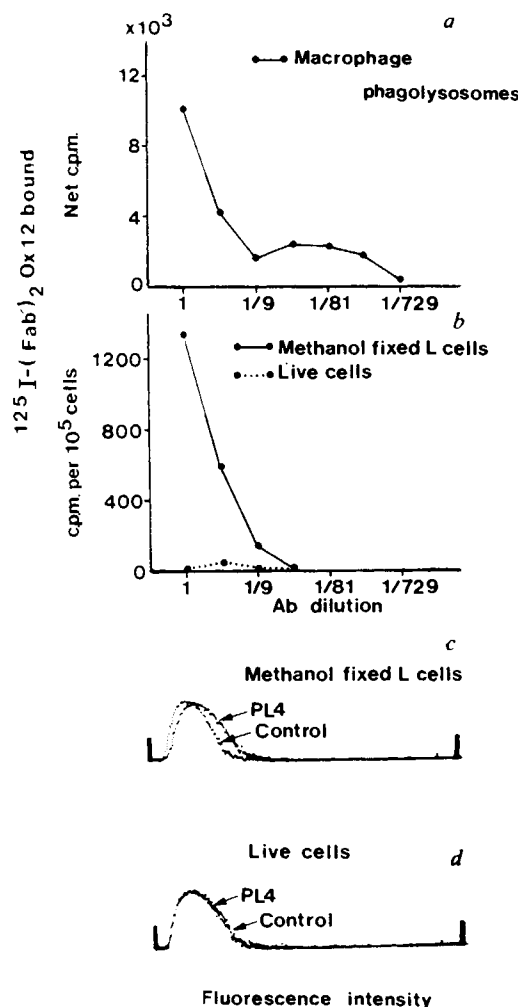


Fig. 1 PL4 detects an intracellular antigen in macrophages and fibroblasts. PL4 cells were grown in Iscove's modified minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), heat inactivated for 30 min at 56 °C, and antibiotics (1+5). The antibody, a rat IgG2a, was precipitated with $(\text{NH}_4)_2\text{SO}_4$, washed, dissolved in phosphate-buffered saline, pH 7.4 (PBS) and dialysed against PBS + 10 mM NaN_3 . **a**, LP were prepared from homogenates of TPM fed 1.1- μm latex microspheres (LB11, Sigma) 15 h previously. RIA was performed²⁰ by incubating LP with serial dilutions of PL4 in PBS + 0.1% (w/v) BSA + 10 mM NaN_3 (PBA) for 1 h at 4 °C. PL were washed twice in PBA, pelleted for 3 min and incubated for 1 h at 4 °C with 3×10^5 c.p.m., ^{125}I -F(ab')₂ fragments of MRC OX12, a monoclonal mouse antibody against the rat heavy chain *b* allotype¹¹ donated by Dr S. Hunt. Samples were washed three times in PBA and LP counted in clean tubes in a Packard γ -counter. Results are given as mean counts bound ($n = 2$, range <10% of mean). **b**, Mouse L929 cells²¹ were fixed in methanol at -20 °C for 6 min or used unfixed after a wash in PBA. Binding of PL4 was done as in **a**. Samples were resuspended in Isoton and the number determined in a Coulter counter. Results show mean counts bound per 10^5 cells ($n = 2$, range <10% of mean). **c**, **d**, Live and methanol-fixed L929 cells were incubated with PL4 and then with tetramethylrhodamine conjugated OX12, 25 μg per ml. After washing, cells were examined using a fluorescence activated cell sorter (Becton Dickinson). The profiles display relative number against rhodamine fluorescence. Cell fragments and large aggregates were excluded electronically.

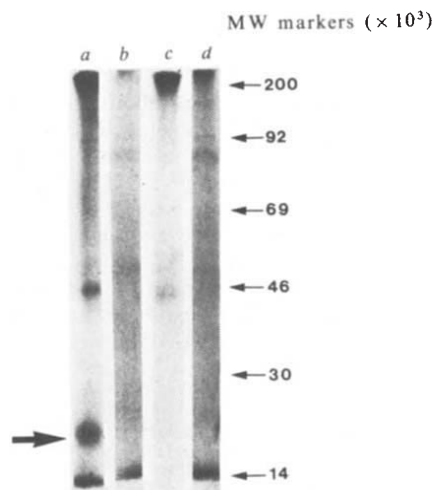


Fig. 2 PL4 precipitates a phosphorylated protein after phagocytosis. TPM were incubated for 1 day at 5×10^6 cells per 100-mm tissue culture dish (Corning 25020) in 5 ml of I+5 (see Fig. 1 legend). Adherent cells were washed twice in saline and 3 ml of MEM with 5% heat-inactivated FBS which was phosphate free, and 1 mCi per dish of $^{32}\text{P}_i$ (Amersham International). Dishes were incubated for 5 h to ensure equilibration of the phosphate pool (trichloroacetic acid-precipitable counts plateau by 90 min), the $^{32}\text{P}_i$ -containing medium was then aspirated and cells were washed with saline. Dishes were treated with 5 ml prewarmed I+5 with or without 1% latex and incubated at 37°C for 15 min. Cells were washed three times with ice-cold PBS with 10 mM NaN_3 and collected with a rubber policeman in 5 ml of ice cold collection buffer (PBS with 1% (v/v) aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM EDTA and 10 mM NaN_3). Cells were centrifuged at 1,000 r.p.m. for 5 min and the pellet dissolved in 1 ml lysis buffer (PBS with 0.5% (v/v) NP40, 0.1% (w/v) SDS, 1% (v/v) aprotinin, 1 mM PMSF and 10 mM NaN_3) on ice, for 15 min. The lysate was clarified by centrifugation for 15 min and distributed to microfuge tubes for immunoprecipitation. 400 μl of lysate was incubated at 4°C for 1 h with 50 μl PL4 and then 25 μl of hyperimmune rabbit anti-rat IgG serum, heat inactivated at 56°C for 30 min and clarified by ultracentrifugation before use. The precipitate was collected by centrifuging for 10 min, washed three times in lysis buffer, and resuspended by sonication in 25 μl of 8 M urea, 5% (w/v) SDS in 25 mM Tris-HCl, pH 7.0. SDS-containing sample buffer (25 μl) was added, and the samples were boiled and analysed on 10% acrylamide SDS-polyacrylamide gels²². Gels were processed for fluorography²³, dried by vacuum and autoradiographed with Kodak X-Omat paper at -70°C for 18 days. Immunoprecipitates: a, PL4, after phagocytosis. b, PBA, after phagocytosis. c, PL4, no phagocytosis. d, PBA, no phagocytosis. ^{14}C -methylated protein markers (Radiochemical International) were run at the same time.

to detect proteins which bind $^{45}\text{Ca}^{2+}$. Figure 3 shows the major Ca^{2+} -binding species in a lysate prepared from TPM which have not received a phagocytic stimulus. When PL4 is used to immunoprecipitate unlabelled macrophage lysates insufficient protein is recovered to stain with Coomassie blue. However, after blotting and incubation with $^{45}\text{Ca}^{2+}$ it is clear that the antigen recognized by PL4 is a Ca^{2+} -binding protein (Fig. 3d) as well as a target for phosphorylation (Fig. 2a). Similar preparations of other monoclonal antibodies or heterogeneous antisera against LP do not precipitate this molecule. This novel method for detecting Ca^{2+} -binding proteins will reveal only those proteins with an affinity for Ca^{2+} below that of EDTA (otherwise EDTA will not release the unlabelled Ca^{2+} before replacement with $^{45}\text{Ca}^{2+}$) and has the disadvantage common to all nitrocellulose blotting techniques of reduced transfer efficiency with increasing molecular weight. However, the method can detect Ca^{2+} -binding proteins with molecular weight (MW) $>100,000$ (Fig. 3a).

Calcium-binding proteins of similar size have been isolated from various sources including calmodulin¹³, which is not phosphorylated, and myosin light chain¹⁴ which can be phosphorylated in certain conditions. We have found with the aid of sensitive radioimmunoassays that PL4 does not bind to highly purified vertebrate calmodulin¹⁵ or myosin light chains from bovine thymus or chicken gizzard¹⁶. As this antigen is neither species nor tissue specific we suspect that it is a different protein,

but further studies with affinity-purified material are needed to establish its identity.

The continuation of phagocytosis in the absence of extracellular calcium¹⁷ and the lack of muscle-like regulatory components of actomyosin have been used to argue against a role for Ca^{2+} in coupling ligand-receptor binding with actomyosin contraction in the macrophage. However, more recently it has

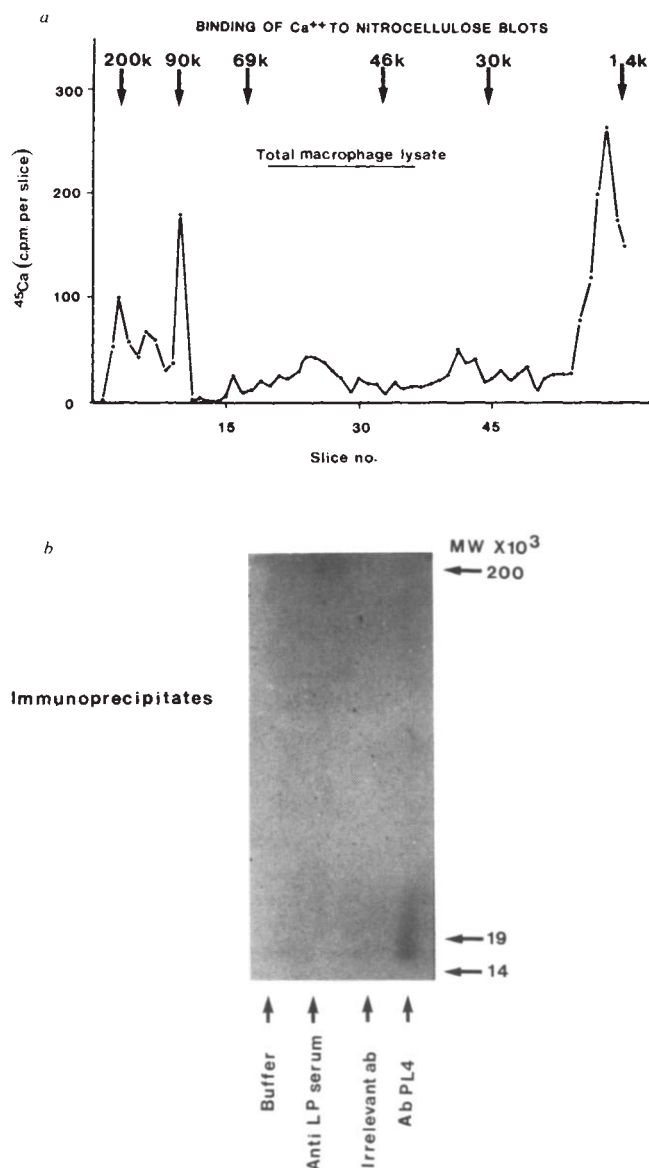


Fig. 3 The protein immunoprecipitated by PL4 binds Ca^{2+} . Unlabelled TPM were prepared without a phagocytic stimulus, as in Fig. 2. Total lysates and PL4 immunoprecipitates were processed and electrophoresed and the gel blotted on nitrocellulose filters by diffusion in transfer buffer (50 mM NaCl, 2 mM NaEDTA, 0.1 mM dithiothreitol in 10 mM Tris-HCl, pH 7.0) for 48 h²⁴. The filters were washed overnight at room temperature in PBS containing 10 mM NaEDTA, made in deionized water, and in three changes of deionized water (1 h each). Filters were incubated with $1 \mu\text{Ci cm}^{-2}$ $^{45}\text{CaCl}_2$ (Amersham International) in deionized water for 3 h at room temperature. Excess $^{45}\text{Ca}^{2+}$ was removed by washing in PBS. The filters were air dried and either autoradiographed with an intensifier screen (Fuji) using Kodak NST-1 film at 4°C for 15 days, or cut into 1-mm strips and counted in a Packard scintillation spectrometer. a, $^{45}\text{Ca}^{2+}$ -binding proteins of the macrophage lysate. Although it is possible to blot ^{14}C -methylated molecular weight markers, in this experiment the nitrocellulose blot was calibrated from markers run on an adjacent unblotted section of the parent gel. b, $^{45}\text{Ca}^{2+}$ -binding proteins after immunoprecipitation with PL4 or controls.

become clear that the macrophage does possess a molecule (gelsolin, MW ~93,000) which confers Ca^{2+} dependence on the contractile system⁶, and that this component is present at the site of phagocytosis (H. L. Yin, personal communication). It is therefore possible that the macrophage contractile machinery is ultimately under Ca^{2+} control, as in skeletal muscle¹⁸. If this is the case, then the lack of requirement for extracellular Ca^{2+} suggests that the local increased Ca^{2+} is due to some event other than an inward Ca^{2+} flux across the plasma membrane. The Ca^{2+} may be released into the cytosol from some intracellular site of sequestration, triggered by ligand-receptor binding at the cell surface. The component of the macrophage described here is a good candidate to mediate such a calcium signal and experiments are now under way to examine the kinetics of

phosphorylation and its effect on the Ca^{2+} -binding affinity of the molecule.

The antigen detected by PL4 is present in cells before phagocytosis and binds Ca^{2+} in this form, although incorporation of ^{32}P occurs only after a phagocytic stimulus. Nonphysiological stimuli which perturb the membrane such as phorbol myristate acetate¹⁹ also have this effect on the antigen. Further studies are needed to define the association between this antigen and the phagolysosomal membrane and its relation to phosphorylation by phagocytosis.

We thank Ms Maxine Bampton for technical help and Drs D. M. Watterson and J. Kendrick-Jones for purified calmodulin and myosin light chains. This work was supported by a research studentship and a grant from the MRC.

Received 24 December 1981; accepted 8 July 1982.

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Dopaminergic supersensitivity induced by denervation and chronic receptor blockade is additive

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A persistent, severe reduction of synaptic transmission has been shown to enhance the responsiveness of postsynaptic tissue to the deficient neurotransmitter—a phenomenon called postjunctional supersensitivity^{1–4}. Regardless of whether supersensitivity is induced by removal of presynaptic nerve terminals (denervation) or by chronic postsynaptic receptor blockade, qualitatively similar adaptations in peripheral autonomic targets are usually produced, suggesting that the two types of manipulation are interchangeable and that the postsynaptic molecular alterations they produce are identical². Following bilateral destruction of dopamine-containing afferents to the nucleus accumbens in the rat, there is a pronounced behavioural hyperresponsiveness to direct dopamine agonists^{5–7}. A remarkably similar situation develops after chronic administration of antipsychotic drugs^{8–10}, such as haloperidol, which are known to be potent antagonists at putative dopamine receptors^{11–13}. We report here that a combination of these two procedures unexpectedly leads to far greater dopaminergic supersensitivity than is observed with either treatment alone. In subjects with the combined treatments, the dopamine agonist apomorphine was more effective in tests of locomotor activation, and additive elevation of the B_{max} for ^3H -spiperone binding in the nucleus accumbens was observed. These results suggest that denervation and chronic exposure to receptor antagonists can lead to independent processes of postjunctional supersensitivity.

Male Sprague-Dawley rats (Charles River Laboratories; mean weight 305 g) were used. To study denervation supersensitivity, 8 μg of 6-hydroxydopamine were injected into the nucleus accumbens on each side as previously described¹⁴. Sham-treated control subjects were prepared with bilateral

injections of the ascorbate/Ringer's 6-hydroxydopamine vehicle. On the third post-surgical day, the animals were screened for dopaminergic behavioural responsiveness to apomorphine (0.1 mg per kg = 329 nmol free base per kg; method as in Fig. 1 legend). The cumulative counts were used to rank 6-hydroxydopamine- and sham-injected subjects into two equally responsive groups of the same size.

On the day following this screening, chronic drug treatment was initiated. Haloperidol (McNeil Injectable), 2.2 μmol per kg per day, was administered subcutaneously (s.c.) to one-half of the 6-hydroxydopamine-treated and one-half of the sham-treated animals for 14 (expt 2) or 15 (expt 1) days. The control animals were injected with haloperidol vehicle¹⁵ (see Fig. 1 legend for details). None of the treatments resulted in any overt toxicity.

Seventeen days after the lesions, the rats were tested for locomotor activation by various doses of apomorphine-HCl (32.9–329 nmol per kg, s.c.). On the day following the final behavioural assessment, the animals were decapitated and catecholamine levels in the nucleus accumbens were determined (see Fig. 2 legend).

A second series of animals (expt 2) were treated for analysis of nucleus accumbens neuroleptic-binding sites. The animals were evaluated for apomorphine-induced locomotor activity (0.1 mg per kg, s.c.) as detailed above on the second day of withdrawal from chronic drug administration. On the following day, the subjects were decapitated and ^3H -spiperone-binding sites in the nucleus accumbens were measured¹⁶.

Figure 1 shows the results of behavioural testing with apomorphine-HCl at one dose (165 nmol per kg). In both sham-lesioned animals chronically treated with haloperidol and animals receiving 6-hydroxydopamine injections and vehicle, apomorphine potentiated locomotor activity ($P < 0.01$ and $P < 0.001$ respectively). When treatment with 6-hydroxydopamine and chronic haloperidol injections were combined, the response to apomorphine was considerably greater than with either treatment alone. Spontaneous locomotor activity before apomorphine injection was not significantly different between groups. The same pattern of response was observed at all doses of apomorphine (Fig. 2). There were no significant effects of the saline/ascorbate vehicle.

Additional information was obtained by determining the ED_{50} values, defined as the dose of apomorphine which led to

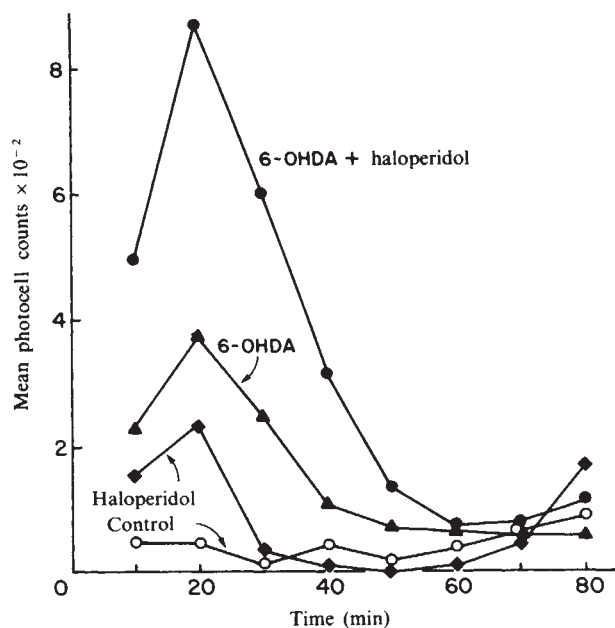


Fig. 1 Locomotor activity following interscapular subcutaneous injection (at $t=0$ min) of 165 nmol per kg apomorphine-HCl. Before injection, the animals were allowed a 70–80 min habituation period in the testing cages, during which time no effects of the various chronic pretreatments were observed. Activity was evaluated in cages containing twin photocell beams as previously described¹⁵. The values are the mean photocell counts recorded during each preceding 10 min interval for the groups represented as follows: $\circ$, bilateral sham nucleus accumbens injections +15 days of haloperidol vehicle ($n=7$); $\blacklozenge$, sham nucleus accumbens lesion +15 days of 1 mg per kg haloperidol s.c. ($n=8$); $\blacktriangle$, bilateral 6-hydroxydopamine (6-OHDA; 8 μ g each side) nucleus accumbens injections +15 days of haloperidol vehicle ($n=6$); $\bullet$, 6-hydroxydopamine nucleus accumbens lesions +15 days of haloperidol ($n=8$). Statistical analysis was accomplished with two-factor analysis of variance, which allowed the testing for main effects of chronic haloperidol and 6-hydroxydopamine treatment as well as for their interaction, with significance assumed in each case when $P<0.05$.

a doubling of the cumulative activity scores in one-half of the subjects in each group compared with the control group. In animals receiving prolonged haloperidol treatment alone, ED_{50} was achieved in the range 82–165 nmol apomorphine per kg, while that for subjects receiving only 6-hydroxydopamine was 33–82 nmol per kg of apomorphine. In the case of the combined treatment, the apparent ED_{50} was observed with 33 nmol apomorphine per kg. The rats receiving the combined treatment were approximately twofold more sensitive to the locomotor activating effect of apomorphine than those with the lesion alone and approximately fivefold more sensitive than those receiving chronic haloperidol alone. Moreover, our data (Fig. 2) clearly demonstrate that the magnitude of locomotor activation induced by 165 and 329 nmol per kg of apomorphine in subjects with the combined treatment is greater than the sum of the apomorphine-induced activation seen with either treatment alone. Thus, the potency and efficacy of apomorphine were both enhanced in animals with the combined treatment compared with those with either treatment alone.

Figure 3 shows a part of a Scatchard¹⁶ analysis of 3H -spiperone binding to membranes prepared from the nucleus accumbens of the similarly pretreated groups of expt 2. Statistical analysis revealed two significant effects—an elevation of B_{max} values produced by treatment with the neurotoxin ($P<0.001$) and a separate elevation produced by chronic administration of haloperidol ($P<0.001$). There was no significant interaction of 6-hydroxydopamine and the chronic neuroleptic. In contrast, we could not detect any effect of the pretreatments on the K_d values ($P>0.05$). In animals with

bilateral injections of 6-hydroxydopamine into the nucleus accumbens and chronic haloperidol administration, there was a greater increase in the density of 3H -spiperone-binding sites than there was with either treatment alone, and, again, the affinity constant was not altered. The quantitatively similar effects of 6-hydroxydopamine and chronic haloperidol treatments on the B_{max} (both producing ~15% increase) suggested that, together, the two treatments can produce an additive increase in the receptor density.

Our findings confirm previous reports that enhanced apomorphine-induced locomotor activity follows bilateral 6-hydroxydopamine injection into the nucleus accumbens region, a phenomenon which is thought to signify post-junctional dopaminergic supersensitivity in mesolimbic targets^{6,7,17}. However, until the present study, there has been no corresponding report of an increased density of 3H -spiperone-binding sites.

Our most intriguing result is the enhancement, which is at least additive, of apomorphine-induced locomotor activity when near-total elimination of dopamine from the nucleus accumbens is coupled with prolonged exposure to haloperidol. This finding is not predicted on the basis of classical investigations of supersensitivity², because both denervation and chronic receptor blockade are thought to evoke post-junctional supersensitivity by the same route—by persistently preventing neurotransmitter action at the postsynaptic membrane. Indeed, one interpretation of our results is that chronic exposure of postsynaptic elements in the nucleus accumbens to the antagonist in the absence of any appreciable neurotransmitter could, in its own right, trigger some of the adaptations comprising supersensitivity. This is supported by the independent elevations of nucleus accumbens dopaminergic (D-2)¹⁸ receptor densities secondary to denervation and long-term receptor

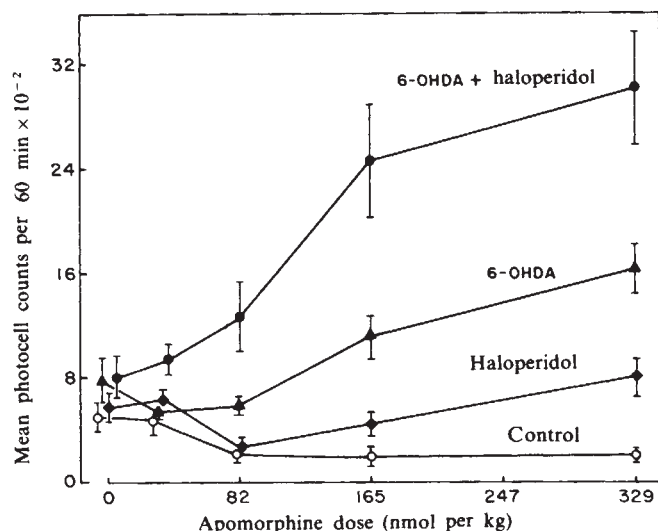


Fig. 2 Effect of various doses of apomorphine-HCl on locomotor activity. Note that the dose 329 nmol per kg corresponds to 0.1 mg per kg apomorphine-HCl. Values are the cumulative number of photocell beam interruptions (mean $\pm$ s.e.m.) for the 60 min following apomorphine administration. Symbols and number of subjects in each group as in Fig. 1 legend. Subjects were allowed to habituate to the testing apparatus for 60–90 min before apomorphine injection. One day after the behavioural tests were completed, all subjects were decapitated for analysis of dopamine and metabolite levels in nucleus accumbens. The nucleus accumbens were dissected from a coronal slice over ice as described previously²² (wet wt ~8 mg) and immediately homogenized in 0.5 ml (~60 vols) of 0.1 M $HClO_4$ (containing 1 M $NaHSO_3$). After sonication, aliquots of each sample were centrifuged and purified by elution from alumina columns. Analysis was completed using HPLC (for details, see ref. 23). Dopamine levels (ng per mg protein) were as follows: control, 62.4 ± 5.6 ; haloperidol, 63.7 ± 3.9 ; 6-hydroxydopamine, 1.33 ± 0.21 (98% loss compared with control); 6-hydroxydopamine + haloperidol, 1.85 ± 0.52 (97% loss compared with haloperidol).

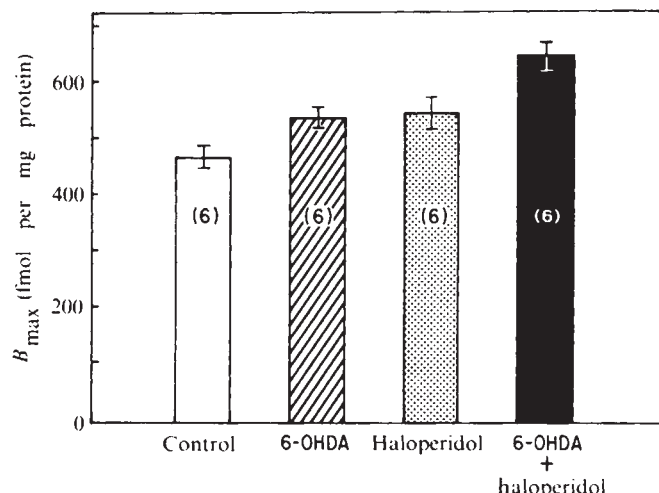


Fig. 3 Density of nucleus accumbens ³H-spiperone-binding sites as a function of treatment history. The nucleus accumbens were isolated (Fig. 2 legend) separately for each animal, homogenized in 1.5 ml of 10 mM Tris-maleate (with 0.9% NaCl, pH 7.5) and spun for 10 min at 20,000g at 4 °C. Pellets were resuspended in 12 ml (mean final protein concentration, 60.0 µg per 900 µl) of 20 mM Tris buffer (with 0.9% NaCl, pH 7.5) using a Polytron (setting 5). An aliquot of the suspended membranes (900 µl) was combined with 100 µl of a solution containing ³H-spiperone (final concentrations 0.08–1.5 nM; six points in singlet for each subject) or the radioligand plus (+)butaclamol (final concentration 2 µM) to define the nonspecific binding. Further details of the binding procedure are described elsewhere²⁴. Average specific c.p.m. bound in control group binding curves were 167 and 669 for 0.08 and 1.5 nM ³H-spiperone respectively. B_{max} (corrected for protein²⁵) and K_d values were determined for each animal using Scatchard analysis¹⁶ and linear regression (correlation coefficients were 0.86–0.99). Control K_d values were 254 ± 24.1 pM. A recent investigation²⁶, including pharmacological characterization of ³H-spiperone-binding sites in the nucleus accumbens-olfactory tubercle and regional study of chronic neuroleptic-induced increases in the density of those sites strongly suggest that nucleus accumbens membrane sites labelled by this radioligand are of the dopaminergic D-2 class¹⁸ that has been characterized in the neostriatum. Thus, the nucleus accumbens sites are chiefly non-serotonergic^{27,28}. Pretreatments are as described in Fig. 1 legend, except that haloperidol was administered for 14 days. Values are the mean $\pm$ s.e.m., n in parentheses.

blockade (see Fig. 3 legend for comments on ³H-spiperone binding in nucleus accumbens). It is interesting to note that acute haloperidol administration was found to decrease acetylcholine content in the corpus striatum after the elimination of the dopaminergic input¹⁹.

Because haloperidol is known to increase dopamine release which could, in part, compensate for postsynaptic receptor blockade, it could be argued that chronic haloperidol by itself does not lead to maximal supersensitivity and that dopaminergic denervation merely gives rise to the full expression of haloperidol-induced supersensitivity. However, this explanation is extremely unlikely. Because dopamine and haloperidol compete for postsynaptic receptor sites, a greater daily dose of haloperidol might be expected to produce greater postsynaptic receptor blockade and, thus, greater supersensitivity, if other factors were equal. However, it was demonstrated²⁰ that a substantially higher dose of haloperidol than we used did not produce a greater elevation of dopamine receptors, and, furthermore, haloperidol-induced dopamine utilization in rat nucleus accumbens has been shown to be reduced as the magnitude of the repeated doses is raised over a 10-fold range (0.5–5 mg per kg)²¹. Thus the idea that haloperidol-lesion-induced postsynaptic blockade is sub-maximal due to the ability of the antagonist to elevate dopamine release is unsupported.

The marked enhancement of behavioural supersensitivity following combined denervation and chronic receptor blockade in our studies is therefore probably the result of potentiated

postjunctional supersensitivity phenomena in the nucleus accumbens. Furthermore, the increase in receptor density induced by haloperidol in 6-hydroxydopamine-treated rats cannot be located on presynaptic dopamine terminals, demonstrating that the supersensitive response to both treatments must be located on postsynaptic elements. It will be interesting to investigate the generality of these phenomena by analogous testing in other neurotransmitter systems.

We thank N. Callahan for preparation of the manuscript, Drs A. Ettenberg and S. Young for helpful discussion of data analysis, and H. Pettit and L. Randolph for technical assistance. D.A.S. was supported by grant MH 08080, P.J.M. by Swiss NSF fellowship 83.832.0.80 and W.J.S and F.E.B. by NIMH grant 29466.

Received 24 February; accepted 1 July 1982.

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Enkephalin opens potassium channels on mammalian central neurones

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Enkephalin and opiate analgesic drugs exert their effects on the brain by interacting with receptors located on neuronal membranes. An almost immediate consequence of this interaction is an inhibition of action potential discharge of individual nerve cells¹. This could result from a direct hyperpolarization of the neurone which bears the opiate receptors, thereby shifting its membrane away from the threshold for action potential generation. On the other hand, it is well known that opiates and enkephalin depress the release of neurotransmitters^{2,3}, and this could indirectly result in an inhibition of cell firing. Here we describe experiments which indicate that Met⁵-enkephalin and a stable analogue, D-Ala²-D-Leu⁵-enkephalin (DADLE), as well as narcotic analgesics, increase the potassium conductance of neurones in the rat locus coeruleus and thereby inhibit their spontaneous firing. This effect of the opioids results from an interaction with a receptor having a high affinity for naloxone.

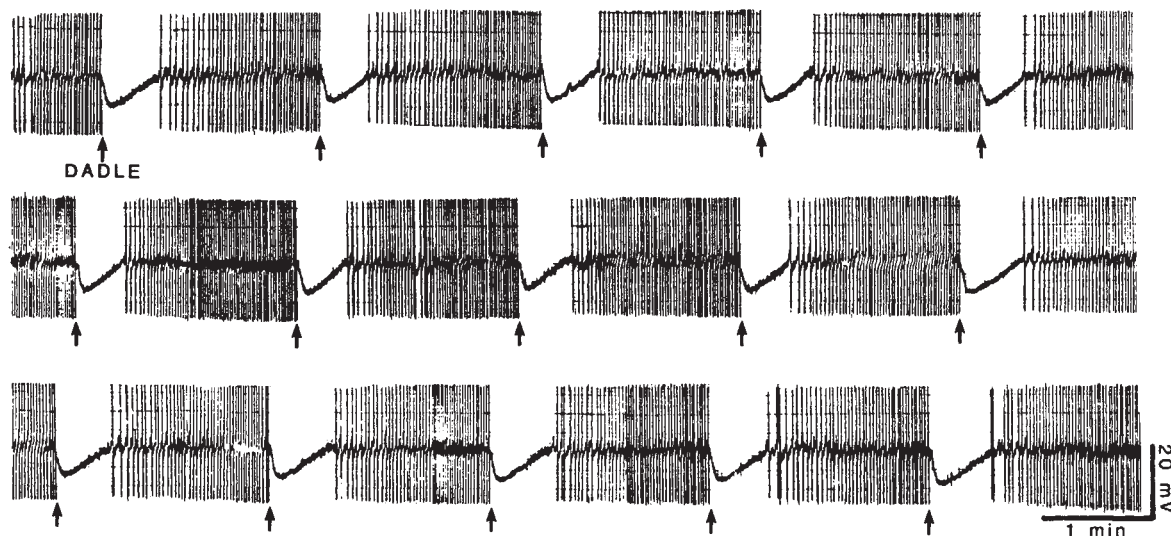


Fig. 1 Enkephalin inhibits cell firing. The figure shows an intracellular recording from a spontaneously firing locus coeruleus neurone. DADLE was applied by a brief (50 ms) pulse of pressure (70 kN m^{-2} , 10 p.s.i.) to a pipette positioned just above the brain slice at the times indicated by the arrows. The cell hyperpolarized and the action potential discharge was inhibited. The pen recorder did not reproduce the full action potential amplitude, which was 70–100 mV among different neurones. These recordings were made from a completely submerged slice of pons (thickness $\sim 300 \mu\text{m}$) continuously superfused with a solution of the following composition (nM): NaCl 126, KCl 2.5, CaCl_2 2.4, NaH_2PO_4 1.2, MgCl_2 1.3, NaHCO_3 25, glucose 11, which was equilibrated at 37°C with 95% O_2 , 5% CO_2 .

We studied the effects of opiates and enkephalin on neurones in the nucleus locus coeruleus. This is a compact group of noradrenaline-containing cell bodies which lies close to the floor of the fourth ventricle of the mammalian brain. Its widespread projections allow it to influence many brain regions, several of which have been implicated in the acute responses to opiates. Moreover, the locus coeruleus of the rat has a high density of opiate-binding sites, and enkephalin-like material has been localized in nerve terminals making synaptic contacts with locus coeruleus neurones⁴. It is known that opiates and enkephalin inhibit the firing of these neurones^{5–7}, and a previous study in the guinea pig indicated that this is due to a membrane hyperpolarization⁸. Intracellular recordings were made from locus coeruleus neurones in $300 \mu\text{m}$ -thick slices of brain tissue cut from the rat pons (see refs 8, 9). Drugs were added to the artificial cerebrospinal fluid solution which continuously superfused the brain slice (see Fig. 1 legend). In addition, normorphine, Met^5 -enkephalin and DADLE were administered by brief application of pressure to a micropipette containing the compound positioned just above the surface of the brain slice.

Most locus coeruleus neurones fired action potentials spontaneously at frequencies of 1–4 Hz, which is similar to their behaviour *in vivo*^{5,6}. Enkephalin and morphine each caused a concentration-dependent inhibition of this firing and a membrane hyperpolarization when added to the solution which superfused the slice (Fig. 1). The minimum concentration which inhibited firing was 10 nM, and the hyperpolarizations (mV, mean $\pm$ s.e.m.) from the resting potential were: for normorphine ($1 \mu\text{M}$), 10.2 ± 1.2 ($n = 12$); normorphine ($10 \mu\text{M}$), 18.3 ± 4.4 ($n = 3$); DADLE (300 nM), 7.8 ± 1.8 ($n = 6$); and DADLE ($1 \mu\text{M}$), 15.6 ± 3.9 ($n = 7$). When the perfusion with morphine was continued for over 1 h the membrane remained hyperpolarized throughout, and rapidly recovered its original resting level when the drug was washed out. Application of Met^5 -enkephalin, DADLE or normorphine by the pressure technique gave hyperpolarizations of a more rapid time course which were dose-dependent, and which could be evoked reproducibly over periods of several hours (Fig. 1).

Several findings indicate that this inhibition of firing and hyperpolarization results from an increase in the K^+ ion conductance. (1) The conductance of the cell measured by passing small current pulses was increased during the opioid hyperpolarization (Fig. 2). This increase in conductance occurred

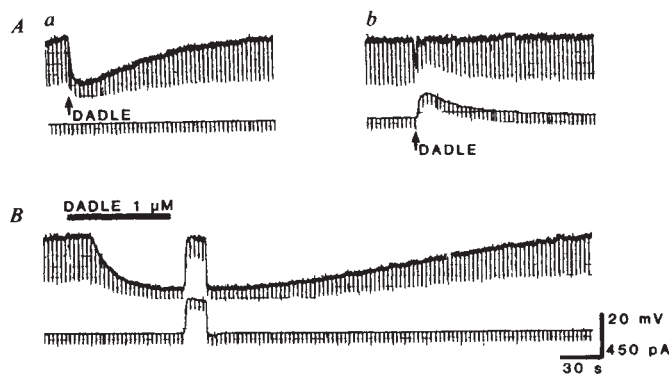


Fig. 2 Enkephalin increases membrane conductance. The upper trace is the membrane potential (resting potential -67 mV); lower trace is the transmembrane current passed by means of a bridge circuit. **A**, DADLE was applied by pressure [two 50-ms pulses, 35 kN m^{-2} (5 p.s.i.)]. In **a**, DADLE hyperpolarized the membrane and reduced membrane resistance. In **b** the DADLE hyperpolarization was annulled by passing inward current. **B**, a similar experiment with superfusion of DADLE ($1 \mu\text{M}$) during the period indicated. The apparent delay in onset of the hyperpolarization is due to passage through the perfusion system. Restoration of the membrane potential to its control level allowed separation of the DADLE-induced resistance change and membrane rectification. In experiments of this type the amplitude of the enkephalin (or normorphine)-induced resistance changes was correlated with the potential change (ΔV) and used to estimate the response reversal potential $E_{\text{rev},R}$ from $\Delta V = (1 - R'/R)(E_{\text{rev},R} - E_m)$ where E_m is the membrane potential before application of enkephalin, R is the control input resistance, and R' is the input resistance at peak enkephalin effect, measured after restoring the potential E_m (as in **b**). $E_{\text{rev},R}$ was -135.5 ± 7.5 (mean $\pm$ s.e.m., $n = 11$) for pressure application, and -124.0 ± 8.1 ($n = 10$) for perfusion. These values were always more negative than those estimated by changing E_m (see Fig. 3). E_{rev} was also estimated from $\Delta V = (1 - \tau'/\tau)(E_{\text{rev},\tau} - E_m)$ where τ' and τ are time constants of small hyperpolarizing current pulses. $E_{\text{rev},\tau}$ was $-103 \pm 5.9 \text{ mV}$ ($n = 10$) with application of opioids by perfusion. $E_{\text{rev},\tau}$ is relatively insensitive to the location of the conductance increase¹⁵, whereas $E_{\text{rev},R}$ will be an overestimate of E_{rev} if the conductance change significantly affects electronically distant membrane. This indicates that enkephalin increases the conductance of both the soma and the dendrites.

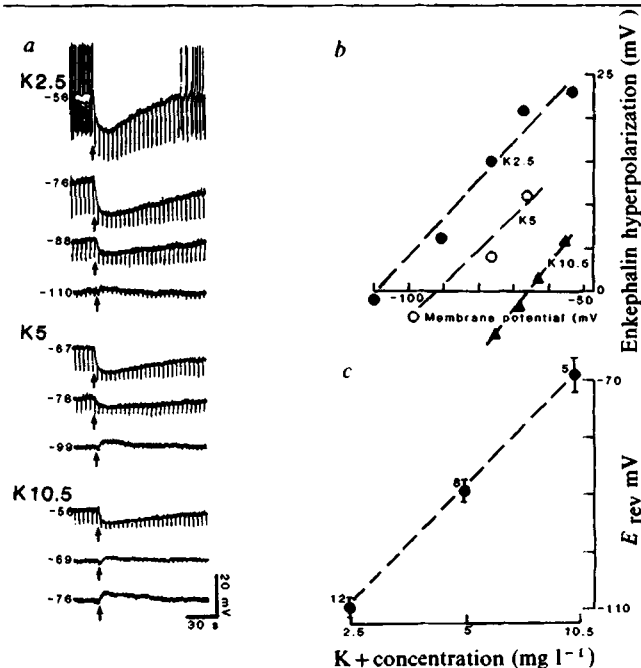


Fig. 3 Enkephalin opens potassium channels. *a* DADLE was applied by pressure [arrow, 50-ms pulse 70 kN m^{-2} (10 p.s.i.)] at various membrane potentials. The potassium concentration was as indicated (K , mM). At elevated K^+ concentrations, the enkephalin response reversed polarity with membrane hyperpolarization. Note the marked rectification occurring with hyperpolarization and with elevated K^+ concentration. *b* Relationship between hyperpolarization amplitude and membrane potential. *c*, Enkephalin reversal potential (E_{rev}) calculated from many experiments such as those in *a* and *b*. The broken line is $E_{\text{rev}} = RT/F \ln([K]_o/[K]_i)$, where $[K]_i$, the intracellular K^+ concentration, was taken as 145 mM. (In normal potassium solutions (2.5 mM), it was usually not possible to reverse the enkephalin hyperpolarization, therefore extrapolated values were used.) The good agreement between the observed values of E_{rev} and those predicted from the Nernst equation suggests a predominantly somatic site of action; the large steady-state conductance increase which accompanied membrane hyperpolarization would effectively remove the dendritic contribution (see Fig. 2 legend).

both on the neurone soma and dendrites (see Fig. 2 legend) (2) The enkephalin hyperpolarization became smaller when it was evoked at hyperpolarized resting membrane potentials, and the response reversed polarity at $\sim -105 \text{ mV}$ (Fig. 3). Complete reversal of the hyperpolarization was often not possible, presumably because of the dendritic site of action combined with strong rectifying properties of the membrane (Fig. 3). The extrapolated reversal potentials followed the K^+ equilibrium potential (E_K) when the extracellular K^+ concentration was altered (Fig. 3). (3) Intracellular injection of Cs^+ or extracellular application of Ba^{2+} both rapidly abolished the opioid hyperpolarizations. (4) The opioids prolonged the after-hyperpolarizations which followed a burst of action potentials; this after-hyperpolarization is due to an increase in K^+ conductance triggered by Ca^{2+} entry during the action potentials (J.T.W. and R.A.N., unpublished observations). (5) Finally, Cl^- movement probably does not contribute to the response because opioid hyperpolarizations were observed equally well with intracellular electrodes containing chloride, methylsulphate or citrate anions, and because chloride-dependent hyperpolarizations induced by γ -aminobutyric acid reversed to depolarizations after a period of recording with chloride-filled electrodes, whereas opiate hyperpolarizations did not change.

The opiate-induced K^+ activation was apparently voltage-dependent: at potentials less negative than the resting potential (which was -55 to -60 mV), the amplitude of the enkephalin hyperpolarization declined sharply. This decrease in effect was not related to the fact that the cells were firing rapidly at such depolarized potentials—similar effects were observed when spontaneous firing was abolished in solutions containing tetrodotoxin and cobalt. This voltage-dependence is similar to that of the K^+ activation by acetylcholine on autonomic ganglion cells¹⁰ and of the K^+ activation by intracellular Ca^{2+} in myenteric neurones¹¹. It allows maximum effect of opiates at membrane potentials close to threshold.

The hyperpolarization induced by opiates was essentially unchanged in calcium-free solutions, as was previously observed^{8,12}. Thus their action is not due to release of other transmitters which then increase the K^+ conductance of the impaled neurone.

The opiate antagonist naloxone reversibly inhibited the action of opiates. Because of the high reproducibility of the

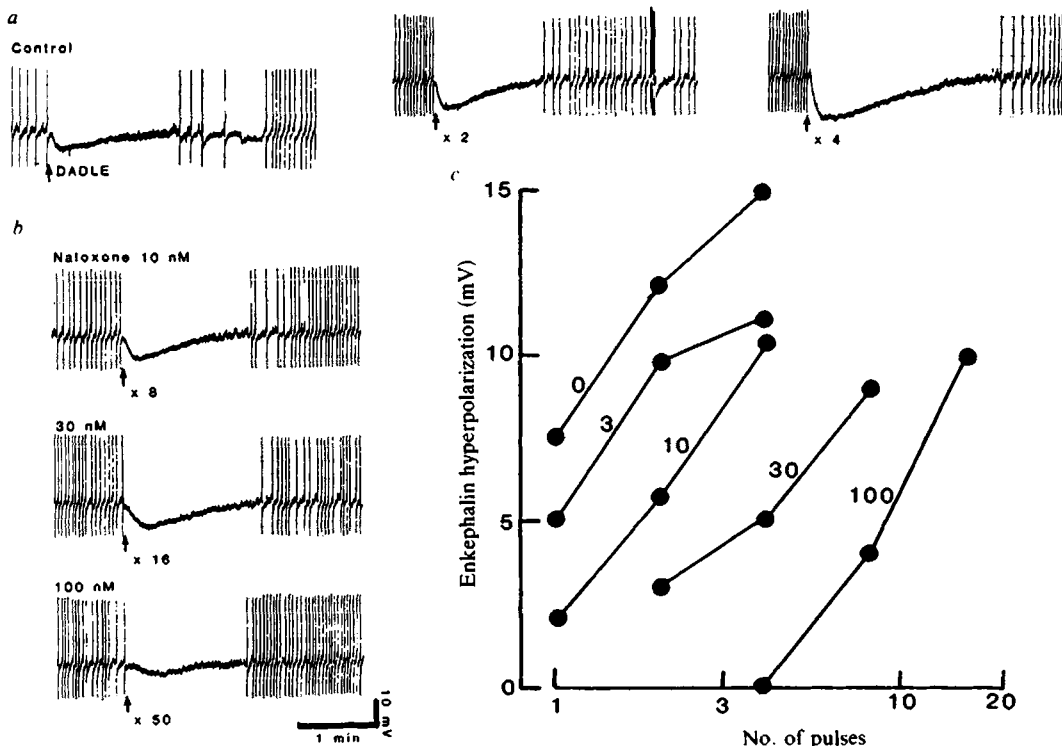


Fig. 4 Enkephalin interacts with naloxone-sensitive receptor. *a*, Control dose-response curve to DADLE applied by pressure (arrow, 24 ms, 55 kN m^{-2} (8 p.s.i.), number of pulses indicated). *b*, Selected records from similar dose-response curves in the presence of various concentrations of naloxone. *c*, Dose-response curves to DADLE at the concentrations of naloxone indicated (nM). A response level was chosen so as to minimize interpolative errors and a Schild¹⁶ plot was used to calculate the dissociation equilibrium constant.

hyperpolarization induced by pressure application, we were able to demonstrate that this action of naloxone was competitive for dose ratios up to at least 64 (Fig. 4). The dissociation equilibrium constant for naloxone determined from such studies was 1.5 nM (95% confidence limits 2.1–1.1 nM, $n = 4$) which is close to that found in radioactive ligand-binding studies with brain membranes prepared by homogenization. This implies that the affinity of the opiate-binding sites for naloxone determined in such biochemical experiments may be close to that found on functioning neurones.

The opening of K^+ channels by opiates may underlie not only the inhibition of cell firing but could contribute to the reduced transmitter release observed in several preparations^{2,3,13}. Transmitter release might be inhibited if the K^+ activation reduced spike duration and resulted in less Ca^{2+} entry, or if the increase in K^+ conductance caused block of impulse propagation at varicosities¹⁴. Which of these two actions predominates may depend largely on the part of the nerve cell involved, the region of the nervous system examined and on the experimental or physiological conditions, but both would contribute to the depression of the nervous system which is the hallmark of opiate action.

This work was supported by US ADAMHA grants DA 03161 (formerly 02241) and DA 03160 (formerly 01730).

Received 21 May; accepted 1 July 1982.

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Predominance of the amino-terminal octapeptide fragment of dynorphin in rat brain regions

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Dynorphin is a 17 amino acid opioid peptide which was originally isolated and characterized from pig neurohypophysis and gut extracts^{1–3}. It contains a leucine-enkephalin (Leu-enkephalin) sequence at the amino terminus and has an unusually potent *in vitro* opiate activity in the guinea pig ileum longitudinal muscle/myenteric plexus preparation^{1,4}. Immunohistochemical studies have shown that perikarya, nerve fibres and terminals widely distributed throughout the central nervous system^{5,6} are immunoreactive for both dynorphin_{1–17} and α -neo-endorphin^{6,7}, another Leu-enkephalin-containing opioid peptide which is structurally related to dynorphin (Fig. 1) and was isolated from hypothalamus⁸. But although the regional distributions of α -neo-endorphin and dynorphin_{1–17} in rat brain, as measured by radioimmunoassay (RIA), are similar, the molar ratio of the two peptides seems to vary greatly from region to

region, with α -neo-endorphin being present in much higher concentrations than dynorphin_{1–17}^{9–11}. We now report that dynorphin_{1–8}, an amino-terminal fragment (Fig. 1), which has only ~3% of the opioid potency of dynorphin_{1–17}, (ref. 4), is present in up to 10-fold higher concentrations in brain than dynorphin_{1–17} immunoreactivity. We further show that dynorphin_{1–8}, but not dynorphin_{1–17}, occurs in approximately equimolar concentrations with α -neo-endorphin in all brain regions examined, suggesting a close biosynthetic relationship between α -neo-endorphin and dynorphin_{1–8}.

The various peptides were detected in acid acetone extracts of rat brain regions with three different highly specific RIAs using antibodies raised against synthetic dynorphin_{1–17}, dynorphin_{1–8} and α -neo-endorphin. All the RIAs were highly specific for their respective antigen. There was no significant cross-reactivity with related peptides or with the peptides recognized by the other RIAs (Table 1).

When extracts from rat brain regions were analysed using the α -neo-endorphin and dynorphin_{1–17} RIAs, the immunoreactivities for the two peptides were found to have a similar distribution; however, α -neo-endorphin immunoreactivity was present in much higher concentrations than dynorphin_{1–17} immunoreactivity and, more important, the molar ratio of α -neo-endorphin to dynorphin_{1–17} was extremely variable among brain regions (Table 2). This finding seemed to be inconsistent with the close biosynthetic relationship between the two peptides suggested by our initial immunohistochemical observations of the two substances in the same neurones^{6,7}.

Table 1 Specificity of RIAs used to detect dynorphin_{1–17}, dynorphin_{1–8} and α -neo-endorphin in extracts of rat brain regions

Synthetic peptide	% Cross-reactivity in RIA for:		
	Dynorphin _{1–17}	Dynorphin _{1–8}	α -Neo-endorphin
Dynorphin _{1–17}	100	<0.001	0
Dynorphin _{1–8}	0	100	0
α -Neo-endorphin	0	0	100
Leu-enkephalin	0	0	0
Dynorphin _{1–6}	0	0	0
Dynorphin _{1–7}	0	0.001	0
Dynorphin _{1–9}	<0.001	0.01	0
Dynorphin _{1–13}	50	0.001	0
Dynorphin _{6–17}	0	0	0
β -Neo-endorphin	0	0	<0.01
α -Neo-endorphin _{1–8}	0	0	0

RIAs were carried out as described in Table 2 legend. The cross-reactivity was calculated based on the amounts of unlabelled peptide needed to obtain a 50% displacement of ¹²⁵I-labelled peptide from the antisera. The highest concentration of unlabelled peptide tested was 1 μ M. When the displacement was <50% at 1 μ M concentration, the cross-reactivity calculations were based on the amount of displacement obtained at this concentration. (Synthetic peptides were solid phase synthesized and purified by Dr J-K. Chang, Peninsula Laboratories.)

Earlier studies, however, had identified an amino-terminal octapeptide fragment of dynorphin, dynorphin_{1–8}, in hypothalamus and posterior pituitary^{12,13}. We reasoned that if dynorphin_{1–8} were a major product in a biosynthetic pathway involving dynorphin_{1–17} as a precursor or intermediate, then by using antibodies to dynorphin_{1–17} we would greatly underestimate the actual amounts of dynorphin-like molecules present because the antibodies would not detect dynorphin_{1–8} (Table 1). When we measured dynorphin_{1–8} immunoreactivity in brain region extracts, it was indeed found that most of the dynorphin-like peptides corresponded to dynorphin_{1–8}-immunoreactive material with up to 10-fold more dynorphin_{1–8} than dynorphin_{1–17} immunoreactivity (Table 2). By comparing the amounts

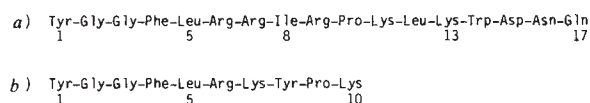


Fig. 1 Amino acid sequences of the structurally related opioid peptides dynorphin_{1–17} (a) and α -neo-endorphin (b) (refs 2, 8).

of dynorphin₁₋₈ and α -neo-endorphin immunoreactivity we concluded that an approximately 1:1 molar ratio of dynorphin₁₋₈ and α -neo-endorphin immunoreactivity exists in all brain regions examined (Table 2).

We estimated the molecular size of the immunoreactive peptides by chromatographing extracts from all brain regions indicated in Table 2 on a Sephadex G-50 column in 50% acetic acid^{14,15}. In addition, immunoreactive peaks from the Sephadex G-50 profile from three regions (posterior pituitary, striatum and midbrain) were rechromatographed by reverse-phase HPLC. Figure 2a-f shows the gel-filtration profiles of hypothalamus (a-c) and striatal (d-f) extracts. In the former

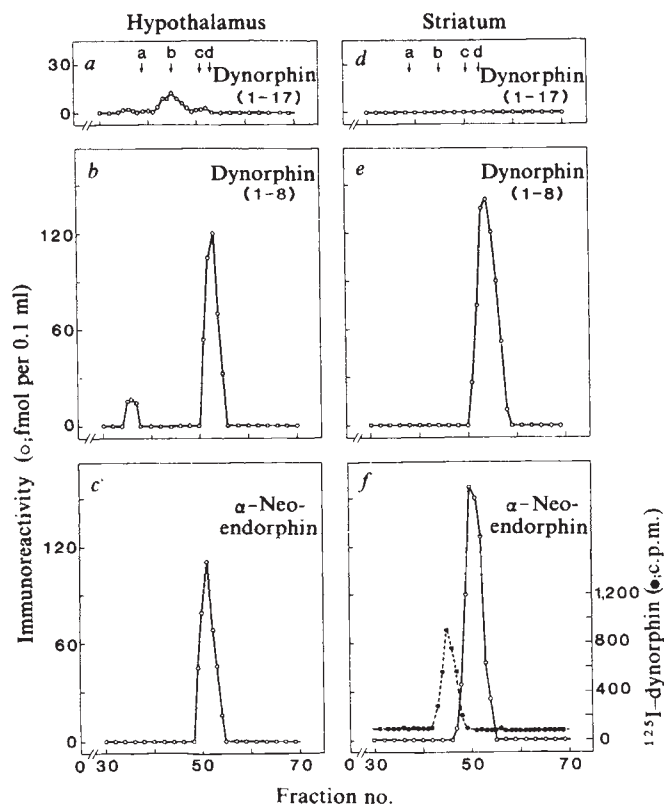


Fig. 2 Gel-filtration chromatography profiles of immunoreactive dynorphin₁₋₁₇, dynorphin₁₋₈ and α -neo-endorphin in rat brain region extracts. Chromatography profiles were obtained from extracts of all brain regions indicated in Table 2. The profiles from hypothalamus (a-c) and striatal (d-f) extracts are shown. The brain regions from 10 animals were extracted with acid acetone as described in Table 2 legend. The supernatants of the acid acetone extracts were subjected to lipid extraction with 10 vol of heptane. In pilot experiments, it was found that ¹²⁵I-labelled dynorphin₁₋₁₇, dynorphin₁₋₈ and α -neo-endorphin did not enter the heptane phase but remained completely in the acid acetone phase. The heptane phases were discarded and the acid-acetone phases evaporated to dryness under a stream of air. The residues were taken up in 1 ml of 50% acetic acid and insoluble material was removed by centrifugation. The supernatants were subjected to gel-filtration chromatography at room temperature on a 120 cm × 0.9 cm column packed with Sephadex G-50 fine resin swollen and equilibrated in 50% acetic acid. Elution of Sephadex G-50 in 50% acetic acid, a strongly dissociating agent, eliminates nonspecific interactions of the peptides with the gel matrix and the elution volume on these columns is a strictly linear function of the logarithm of the molecular weight in the range 500–10,000 (refs 14, 15). Fractions (1.8 ml) were collected and 100- μ l aliquots were evaporated under reduced pressure and assayed in the three RIAs. The columns were calibrated with ¹²⁵I- α -N-acetyl β -endorphin (a), ¹²⁵I-dynorphin₁₋₁₇ (b), ¹²⁵I- α -neo-endorphin (c) and ¹²⁵I-dynorphin₁₋₈ (d). A trace amount of either ¹²⁵I-dynorphin₁₋₁₇ or ¹²⁵I- α -neo-endorphin was included in each run of tissue extract to control the reproducibility of the chromatography. The recovery of immunoreactivity from the columns was 88–92%. To determine whether dynorphin₁₋₈ can be formed from dynorphin₁₋₁₇ as an artefact during extraction, a trace amount of ¹²⁵I-labelled dynorphin₁₋₁₇ was added to the extractant immediately before homogenization of striatum, one of the regions with the largest excess of dynorphin₁₋₈ over dynorphin₁₋₁₇ (Table 2). Figure 1f shows that all the radioactivity from this experiment eluted at the position of ¹²⁵I-dynorphin₁₋₁₇. No radioactivity above background eluted at the position of ¹²⁵I-dynorphin₁₋₈.

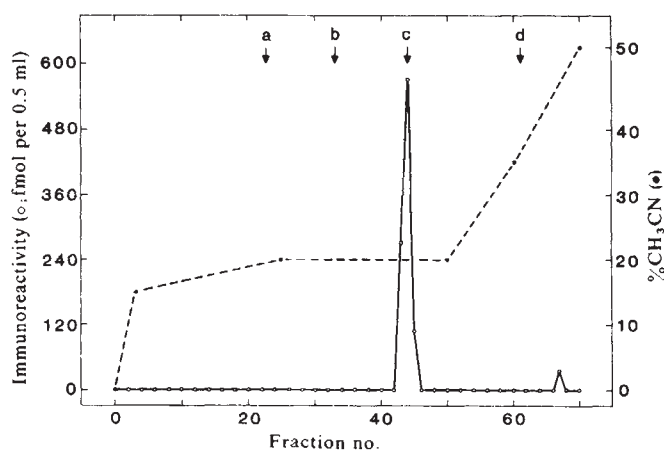


Fig. 3 Reverse-phase HPLC of immunoreactive dynorphin₁₋₈ from rat midbrain extracts. Extracts were prepared and separated on a Sephadex G-50 column in 50% acetic acid as described in Fig. 2 legend. A 500- μ l aliquot from the dynorphin₁₋₈ immunoreactive peak fraction was evaporated under a stream of nitrogen, taken up in HPLC buffer (see below) and chromatographed on an Altex Ultrasphere ODS column (250 mm × 4.6 mm, particle size 5 μ m). Two Altex HPLC pumps and a Beckman gradient mixing computer were used to generate a four-step acetonitrile gradient as shown in the graph. The HPLC buffer consisted of 50 mM monosodium phosphate, 1 mg ml⁻¹ phosphoric acid and 5% methanol, pH 2.7. The flow rate was 1.5 ml min⁻¹ and 1-min fractions were collected. Aliquots (500 μ l) of the fractions were evaporated first under a stream of air to remove the acetonitrile and then under reduced pressure. The residues were assayed in the dynorphin₁₋₈ RIA. Most (>95%) of the immunoreactivity eluted at exactly the same position as synthetic dynorphin₁₋₈ whereas a minor immunoreactive peak of unknown nature eluted at a higher acetonitrile concentration. The co-migration of the immunoreactivity with the synthetic standard was assessed in two ways: (1) the elution of the immunoreactive peak was compared with the elution of the synthetic peptide in a separate run as detected by optical density measurements at 210 nm; and (2) an aliquot from the immunoreactive peak fraction was 'spiked' with an equimolar amount of the synthetic peptide and rechromatographed. In the spiking experiment, the total immunoreactivity eluted as one single peak. When α -neo-endorphin immunoreactive material from the Sephadex G-50 columns was rechromatographed by reverse-phase HPLC we found, as have two other groups^{10,11}, that this material eluted at exactly the same position as synthetic α -neo-endorphin (not shown). An isocratic step was included in the elution profile between fractions 25 and 50 to improve the separation of dynorphin₁₋₈ from β -neo-endorphin. Markers: a, α -neo-endorphin; b, β -neo-endorphin; c, dynorphin₁₋₈; d, dynorphin₁₋₁₇.

extract (a-c), the material detected by the dynorphin₁₋₁₇ RIA constituted only a fraction of the material detected by the dynorphin₁₋₈ or α -neo-endorphin RIA. In the fractionated striatal extracts, dynorphin₁₋₁₇ immunoreactivity was below the detection limits of the various RIAs whereas dynorphin₁₋₈ and α -neo-endorphin immunoreactivities were readily detectable (Fig. 2d-f). For all brain regions, the major immunoreactive peaks for α -neo-endorphin and dynorphin₁₋₈ eluted at exactly the same position as the respective ¹²⁵I-labelled synthetic marker peptides (Fig. 2a-f). Rechromatography by reverse-phase HPLC of the major α -neo-endorphin and dynorphin₁₋₈ immunoreactive peaks from three brain regions (posterior pituitary, striatum, midbrain) showed that the immunoreactivity detected by the α -neo-endorphin and dynorphin₁₋₈ RIAs co-migrated exactly with their respective synthetic marker peptide (Fig. 3).

We conclude that dynorphin₁₋₈ is neither an extraction artefact nor a product of extraneuronal metabolism of dynorphin₁₋₁₇ as (1) when ¹²⁵I-labelled dynorphin₁₋₁₇ was added to the tissue at the time of extraction, no conversion of this radioactive marker to dynorphin₁₋₈ was observed (Fig. 2f); (2) the amounts of dynorphin₁₋₁₇ which we measured are similar to those reported by other groups^{9,11}; and (3) synthetic dynorphin₁₋₁₃ added to washed brain membranes or injected *in vivo* into the brain ventricles is degraded primarily at the amino terminus by aminopeptidases^{16,17}.

The results presented here suggest a precursor-product relationship between dynorphin₁₋₁₇ and dynorphin₁₋₈. It is possible that dynorphin₁₋₈ is derived from a different precursor.

Table 2 Concentration and molar ratios of dynorphin₁₋₁₇, dynorphin₁₋₈ and α -neo-endorphin immunoreactivities in rat brain regions

	Immunoreactivity (pmol per g tissue)			Molar ratios		
	Dynorphin ₁₋₁₇	Dynorphin ₁₋₈	α -Neo-endorphin	D ₁₋₈ /D ₁₋₁₇	α -Neo/D ₁₋₁₇	α -Neo/D ₁₋₈
Medulla/pons	3.9 $\pm$ 0.4	22.5 $\pm$ 0.6	26.3 $\pm$ 1.1	5.7	6.6	1.2
Midbrain	6.2 $\pm$ 1.0	59.6 $\pm$ 3.7	59.7 $\pm$ 2.0	9.6	9.6	1.0
Cerebellum	<0.2	<1.2	<1.7			
Spinal cord	12.1 $\pm$ 2.3	22.7 $\pm$ 1.6	26.5 $\pm$ 2.0	1.9	2.2	1.2
Cortex	2.6 $\pm$ 0.6	21.0 $\pm$ 1.9	19.4 $\pm$ 0.9	8.2	7.6	0.9
Striatum	7.3 $\pm$ 0.7	64.1 $\pm$ 4.1	70.1 $\pm$ 4.5	8.8	9.6	1.1
Hippocampus	3.4 $\pm$ 0.7	26.5 $\pm$ 2.2	25.6 $\pm$ 1.4	7.9	7.6	1.0
Hypothalamus	10.3 $\pm$ 1.1	65.5 $\pm$ 1.8	76.7 $\pm$ 5.7	6.4	7.5	1.2
Posterior pituitary	503.6 $\pm$ 32.9	1384.0 $\pm$ 84.4	1598 $\pm$ 117.9	2.8	3.2	1.1

Brain regions were dissected from 10 male Sprague-Dawley rats (160–180 g) as described in ref. 20. The tissue was frozen immediately on dry ice. After weighing in the frozen state, each tissue sample was individually extracted by sonication in 1 ml of acid acetone (acetone/water 12 M HCl, 40:6:1). The homogenates were centrifuged at 12,000g for 10 min and supernatants were evaporated to dryness under a stream of air in silanized glass tubes. The residue was taken up in 1 ml of RIA buffer and after further centrifugation aliquots of the supernatants were appropriately diluted and assayed in the three RIAs. The RIA procedure was that described in ref. 14, with modifications: the use of a buffer pH of 7.4 rather than 6.0, and application of a double antibody separation procedure rather than a dextran-coated charcoal separation. Antiserum dilutions were 1:130,000, 1:120,000 and 1:30,000 for the dynorphin₁₋₁₇, dynorphin₁₋₈ and α -neo-endorphin RIAs respectively. Values are expressed as corrected means $\pm$ s.e.m. from determinations of the brain regions from 10 individual animals. The measured values were corrected for recovery. The recovery for each peptide from each brain region was determined in separate experiments by adding a trace amount of ¹²⁵I-labelled synthetic peptide to the extractant immediately before homogenization of the sample. After all steps of repeated centrifugation evaporation and rehydration, the amount of recovered radioactivity was 59 $\pm$ 2.3%, 70 $\pm$ 4.1% and 73 $\pm$ 3.3% for dynorphin₁₋₁₇, dynorphin₁₋₈ and α -neo-endorphin respectively. Measurements of the three peptides were repeated twice on two groups of brains from 13 and 6 animals. Brain regions from the 13 animal group were also extracted into acid acetone while the samples from the 6 animal group were extracted into 1 ml each of 0.1 M HCl at 95 °C. The supernatant of the 0.1 M HCl extract was neutralized with an equal volume of 0.1 M NaOH and appropriate dilutions were assayed directly in the RIAs. The results from these two experiments were essentially identical to those shown in the table.

However, previous work showing that α -neo-endorphin and dynorphin₁₋₁₇ immunoreactivities occur within the same neurones^{6,7} and the finding that α -neo-endorphin and dynorphin₁₋₈, but not dynorphin₁₋₁₇, occur in equimolar concentrations throughout the brain suggest that dynorphin₁₋₁₇ is a precursor or intermediate in the formation of dynorphin₁₋₈. A precursor role of dynorphin₁₋₁₇ would explain the rather low concentrations of this peptide in brain. Furthermore, the equimolar distribution of dynorphin₁₋₈ and α -neo-endorphin in all brain regions examined raises the possibility that the two peptides are major products in the processing of a larger common precursor. However, only isolation of such a prohormone or its mRNA can assign a common biosynthetic origin of the two peptides.

The function of dynorphin₁₋₁₇ in brain as an opiate peptide must be re-evaluated in view of the present findings. Dynorphin₁₋₈, the predominant dynorphin-like peptide in brain is far less potent than its putative precursor dynorphin₁₋₁₇ (ref. 4). Given these significant differences in opiate potency of the two peptides, the finding that the ratio of dynorphin₁₋₈ to dynorphin₁₋₁₇ immunoreactivity varies greatly among brain regions, ranging from 2:1 in the spinal cord to 10:1 in the midbrain and striatum (Table 2), may reflect region-specific physiological differences in processing. Certain neuronal populations may leave relatively more dynorphin₁₋₁₇ intact in some regions, thus increasing dramatically the available pool of opiate potency and specificity towards certain receptor types^{18,19}, whereas other neuronal populations may inactivate much of this opiate activity simply by converting dynorphin₁₋₁₇ more completely into dynorphin₁₋₈. Future studies will determine whether external stimuli can significantly alter this processing pattern.

This work was supported by NIDA grant DA 01207 and a Selected Research Opportunity from the ONR (SRO 001:N00014-C-0796). We thank Pamela Angwin and Irene Inman for help with the RIAs and S. Poage for preparing the manuscript.

Received 1 March; accepted 11 May 1982.

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Dynorphin₁₋₈ and dynorphin₁₋₉ are ligands for the κ -subtype of opiate receptor

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It is generally accepted that there are three subtypes of opiate receptor: μ , δ and κ . The main endogenous ligands for the μ - and δ -sites are Met⁵-enkephalin, Leu⁵-enkephalin and β -endorphin, whereas the putative endogenous ligands for the κ -binding site were unknown until recent observations suggested that dynorphin₁₋₁₃ might be a candidate. The most convincing evidence for this view has been presented by Goldstein and his colleagues who showed that dynorphin₁₋₁₃ is a specific endogenous ligand for the κ -receptor and has a high potency and long duration of action¹⁻³. We show here that the sequences Leu⁵-enkephalyl-Arg-Arg-Ile (dynorphin₁₋₈) and, particularly, Leu⁵-enkephalyl-Arg-Arg-Ile-Arg (dynorphin₁₋₉) are selective ligands for the κ -binding site. Whereas dynorphin₁₋₁₃ and dynorphin₁₋₁₇ are relatively resistant to the action of peptidase and have a long duration of action *in vitro* after wash-out, dynorphin₁₋₈ and dynorphin₁₋₉ are readily degraded by peptidases and their duration of action is much shorter. For this and other reasons, the possibility will have to be considered that dynorphin₁₋₈ or dynorphin₁₋₉ may be transmitters or modulators at the κ -binding site while dynorphin₁₋₁₃ and dynorphin₁₋₁₇ may act hormonally, that is, at a distance from the site of release.

Table 1 Inhibitory effects of C-terminally extended Leu⁵-enkephalins on the electrically induced contractions of the guinea pig myenteric plexus-longitudinal muscle and the vasa deferentia of the mouse, rat and rabbit

C-terminal extensions of Leu ⁵ -enkephalin	IC ₅₀ (nM)			
	Guinea pig myenteric plexus	Mouse vas deferens	Rat vas deferens	Rabbit vas deferens
—	28.6 ± 5.4	1.7 ± 0.3	550 ± 62	>10,000
Lys ⁶	107 ± 29.1	25.6 ± 4.9	720 ± 45	>10,000
Arg ⁶	102 ± 17.2	10.9 ± 1.1	790 ± 46	3,000 ± 930
Arg ⁶ -Arg ⁷	17.3 ± 2.52	25.5 ± 4.2	7,500 ± 2,000	43.0 ± 11.5
Arg ⁶ -Arg ⁷ -Ile ⁸	4.92 ± 0.53	9.2 ± 1.30	>10,000	11.7 ± 1.27
Arg ⁶ -Arg ⁷ -Ile ⁸ -Arg ⁹	2.36 ± 0.67	6.5 ± 0.34	>10,000	6.1 ± 0.45
Dynorphin ₁₋₁₃	0.31 ± 0.16	0.33 ± 0.08	>10,000	2.43 ± 0.49
Dynorphin ₁₋₁₇	0.29 ± 0.09	0.91 ± 0.13	>10,000	3.01 ± 1.6
α-Neo-endorphin	2.98 ± 0.44	7.7 ± 0.98	>10,000	21.4 ± 4.5

The values shown are the mean ± s.e.m. of three to four observations. The activity of peptidases has been inhibited by bestatin (10 μM, or 30 μM in the rat and rabbit), L-leucyl-L-leucine (2 mM), thiorphan (0.3 μM) and captopril (10 μM); in the rabbit vas deferens, this treatment increased the potency of the enzyme-sensitive peptides, but not of dynorphin₁₋₁₃ or dynorphin₁₋₁₇, 60–270-fold. The sequence of dynorphin is Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln and of α-neo-endorphin is Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys. The peptides were obtained from Peninsula, Bachem and Cambridge Research Biochemicals or were gifts from Drs A. Goldstein, J. Morley and S. Udenfriend.

Our results were obtained from four *in vitro* pharmacological assays in which the preparations were made to contract by electrical stimulation. The preparations respond differently to activation of the μ-, δ- and κ-receptors. The inhibitory potencies of the peptides are given as their 50% inhibitory concentration (IC₅₀; nM) values. In the myenteric plexus-longitudinal muscle of the guinea pig ileum, an inhibitory effect is readily observed with ligands that activate μ- and κ-receptors, whereas the mouse vas deferens responds preferentially to ligands acting on δ-receptors⁴; the vas deferens of the rat has a low sensitivity to κ-ligands⁵ in contrast to the vas deferens of the rabbit in which κ-ligands, but not μ- or δ-ligands, are inhibitory^{6,7}. In these assays, the activity of peptidases was inhibited by a mixture of bestatin, L-leucyl-L-leucine, thiorphan and captopril (Table 1 and A.T.McK., A.D.C. and H.W.K., unpublished observations). The peptides used in this investigation had agonist, but no antagonist, action.

The binding of the peptides was measured by determining the inhibition constants (K_i, nM) in three assays on homogenates of guinea pig brain. For this purpose, ³H-labelled D-Ala², MePhe⁴, Gly-ol⁵-enkephalin was used as a highly selective μ-ligand and ³H-D-Ala², D-Leu⁵-enkephalin as a δ-ligand which, although selective, has a cross-reactivity of ~10% at the μ-binding site⁸. The most suitable ligand for the assay of the κ-binding site was found to be ³H-(−)bremazocine⁹ after suppression of the binding at the μ- and δ-sites by addition of unlabelled μ- and δ-ligands at a concentration of 100 nM each.

To reduce degradation by peptidases, the binding assays were carried out at 0 °C for 150 min (Table 2).

Two groups of C-terminal extensions of Leu⁵-enkephalin have been shown to occur in the central nervous system (CNS). They are dynorphin₁₋₈ (Leu⁵-enkephalyl-Arg-Arg-Ile; refs 10, 11) and dynorphin₁₋₁₇ (refs 12, 13), which have two arginine residues in positions 6 and 7, and α- and β-neoendorphin^{14,15}, which are nona- and decapeptides respectively, with arginine and lysine residues in positions 6 and 7.

In the pharmacological assays (Table 1), the introduction of Arg⁶ or Lys⁶ at the C-terminal end of Leu⁵-enkephalin reduced its potency without altering the relative activity pattern. However, lengthening the chain from residue 7 onwards increased the ratio of IC₅₀ for the mouse vas deferens to IC₅₀ for the myenteric plexus. The most important effects of the extensions by Arg⁶-Arg⁷ or Arg⁶-Lys⁷ and beyond are the complete loss of potency in the rat vas deferens, which probably has no κ-binding sites⁵, and the progressively increasing activity in the rabbit vas deferens which responds to ketazocine- or κ-like compounds but not to μ- or δ-like ligands^{6,7}. The most potent C-extended forms of Leu⁵-enkephalin are dynorphin₁₋₁₃, dynorphin₁₋₁₇ and dynorphin₁₋₉, Leu⁵-enkephalyl-Arg-Arg-Ile-Arg (Table 1).

These findings are confirmed by the determination of the K_i values at the μ-, δ- and κ-binding sites (Table 2). All C-terminal extensions have lower ratios of K_i at the δ-binding site to K_i at the μ-binding site than the ratio found for the parent Leu⁵-

Table 2 The inhibitory effects of C-terminally extended Leu⁵-enkephalins on the binding in homogenates of guinea pig brain of ³H-D-Ala², MePhe⁴, Gly-ol⁵-enkephalin (1.0 nM), ³H-D-Ala², D-Leu⁵-enkephalin (1.0 nM) and ³H-bremazocine (0.30 nM)

C-terminal extensions of Leu ⁵ -enkephalin	K _i (nM)		
	³ H-D-Ala ² , MePhe ⁴ , Gly-ol ⁵ -enkephalin (μ-site)	³ H-D-Ala ² , D-Leu ⁵ -enkephalin (δ-site)	³ H-(−)bremazocine after suppression of μ- and δ-binding (κ-site)
—	18.8 ± 1.77	1.18 ± 0.20	8,210 ± 1,090
Lys ⁶	12.3 ± 1.91	20.4 ± 2.42	830 ± 118
Arg ⁶	17.4 ± 1.78	10.3 ± 1.11	226 ± 14
Arg ⁶ -Arg ⁷	3.45 ± 0.11	4.45 ± 0.45	9.4 ± 1.01
Arg ⁶ -Arg ⁷ -Ile ⁸	3.83 ± 0.36	4.99 ± 0.86	1.34 ± 0.23
Arg ⁶ -Arg ⁷ -Ile ⁸ -Arg ⁹	3.64 ± 0.64	3.24 ± 0.69	0.209 ± 0.035
Dynorphin ₁₋₁₃	0.222 ± 0.035	0.485 ± 0.076	0.045 ± 0.010
Dynorphin ₁₋₁₇	0.73 ± 0.09	2.38 ± 0.61	0.115 ± 0.018
α-Neo-endorphin	1.24 ± 0.20	0.57 ± 0.086	0.196 ± 0.044

The values shown are the mean ± s.e.m. of three to four observations. The method used for the binding assays has been described previously⁹; incubation was for 150 min at 0 °C. ³H-(−)bremazocine (Dr Römer, Sandoz) was used instead of ³H-(±)ethylketazocine and the binding at the μ- and δ-sites was suppressed with 100 nM each of D-Ala², MePhe⁴, Gly-ol⁵-enkephalin (Dr Römer, Sandoz) and D-Ala², D-Leu⁵-enkephalin (Dr Wilkinson, Wellcome). ³H-D-Ala², MePhe⁴, Gly-ol⁵-enkephalin and ³H-D-Ala², D-Leu⁵-enkephalin were obtained from Amersham.

enkephalin. The most interesting observation, however, is the progressive increase in the affinity at the κ -binding site as eu⁵-enkephalyl-Arg⁶-Arg⁷ (dynorphin₁₋₇) is extended by Ile⁸ (dynorphin₁₋₈) and by Ile⁸-Arg⁹ (dynorphin₁₋₉). Dynorphin₁₋₁₃ as the highest relative affinities at all three binding sites, the values being of an order similar to those described recently for the inhibition of the binding of ³H-dihydromorphine, ³H-D-leu⁵-enkephalin and ³H-ethylketazocine by dynorphin₁₋₁₃ amide in guinea-pig brain³ or inhibition of the binding of ³H-ethylketazocine or ³H-diprenorphine by dynorphin₁₋₁₃ or dynorphin₁₋₁₇ in human brain¹⁶.

The findings with dynorphin₁₋₇, dynorphin₁₋₈ and particularly dynorphin₁₋₉ indicate that these fragments are potent and increasingly selective κ -ligands and therefore merit further analysis, particularly as dynorphin₁₋₈ has been identified in porcine hypothalamus¹⁰ and in the neurointermediate pituitary of the rat¹¹, and dynorphin₁₋₁₇ in rat pituitary¹² and in porcine duodenum¹³. The 'small' dynorphins are readily degraded by peptidases, an action which can be prevented by the mixture of peptidase inhibitors. In the myenteric plexus, the IC₅₀ values of dynorphin₁₋₇, dynorphin₁₋₈ and dynorphin₁₋₉ were, respectively, 367, 30.8 and 21.5 nM before treatment with the peptidase inhibitors and 17.3, 4.9 and 2.4 nM after treatment, an average potentiation of about 12, while there was no potentiation of either dynorphin₁₋₁₃ or dynorphin₁₋₁₇. Furthermore, in four experiments in the presence of the peptidase inhibitors, the recovery of the effect of dynorphin₁₋₉ on the contraction of the myenteric plexus-longitudinal muscle was rapid ($t_{1/2}$ = 48 ± 6 s) and the recovery after dynorphin₁₋₁₇ was slow ($t_{1/2}$ = 178 ± 8 s). Finally, in the rabbit vas deferens pretreated with peptidase inhibitors, the IC₅₀ value of dynorphin₁₋₈ is ~11.7 nM and that of dynorphin₁₋₁₃ 2.43 nM, while the corresponding K_1 values at the κ -binding site are 1.34 and 0.045, respectively. Thus, the 'small' dynorphins are liable to be degraded by peptidases, are easily washed out and have moderate potency at the κ -site while the 'large' dynorphins are peptidase resistant, do not wash out readily and are "extraordinarily potent"¹⁷. The small dynorphins seem to have the qualities of a neurotransmitter or neuromodulator of short and rapid action, whereas the large dynorphins have the characteristics of a highly potent hormonal action of long duration. Moreover, it is of interest that the small dynorphin found in neuronal tissue is dynorphin₁₋₈, which is a less selective κ -ligand than dynorphin₁₋₉. Note that the κ -binding site is very demanding in its choice of ligands as Met⁵-enkephalin, Leu⁵-enkephalin and β -endorphin have only low affinities¹⁸; thus, dynorphin₁₋₈, which has affinities to the μ - and δ -binding sites, is sufficiently selective as far as the κ -binding site is concerned.

An urgent requirement is confirmation or otherwise that dynorphin₁₋₈ and possibly dynorphin₁₋₉ are strong candidates for the role of an endogenous ligand at the κ -binding site mediating neurotransmission or neuromodulation. Lastly, it will be important to correlate the putative κ -like actions of the small dynorphins with the pharmacological effects of the ketazocine-like compounds or κ -agonists^{19,20} which are antinociceptive agents and yet do not substitute for morphine in the morphine-dependent rhesus monkey²¹⁻²³. It may now become possible to exploit the therapeutic potentials of this group of compounds⁸.

This work was supported by grants from the MRC and the US National Institute on Drug Abuse (DA 00662).

Received 17 May; accepted 14 July 1982.

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Direct structural localization of two toxin-recognition sites on an ACh receptor protein

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One of the most actively studied chemoreceptors is the acetylcholine receptor (AChR) in the postsynaptic membranes of neuromuscular junctions and electrocytes. The AChR from *Torpedo* is a membrane protein, each molecule comprising five polypeptide chains^{1,2}: two α -chains of molecular weight (M_r) 40,000, and three homologous chains of M_r 50,000 (β), 60,000 (γ) and 65,000 (δ). Only the α -subunits seem to carry the specific recognition sites for agonists and antagonists². As snake α -neurotoxins seem to bind highly specifically and quasi-irreversibly to these sites we performed a structural analysis to localize the α -subunits, using as marker native α -bungarotoxin (α -BTX, M_r 8,000; ref. 3). We report here the results obtained at 20 Å resolution by electron microscopy and single-particle image averaging⁴, which reveal two well-defined regions within the AChR structure^{5,6} where the mass increases significantly on binding of α -BTX. One of these (α_1) is adjacent to the region where the δ -subunit has been located⁶; the second region (α_2) is diametrically across the molecule, ~50 Å away from the δ -subunit and also from (α_1). The topography revealed by direct structural analysis can explain the results of previous nearest-neighbour cross-linking studies^{2,7}.

AChR-rich membrane fragments isolated from *Torpedo marmorata* electric organ had a specific activity of 1.9 nmol ³H-labelled α -BTX per mg protein. Aliquots (5 μ l; 7 mg protein per ml) were incubated for 60 min with 45 μ l of H₂O which had been adjusted to pH 11 by NaOH, then 120 μ l of H₂O were added and the aliquots were centrifuged in a Beckman Airfuge at 20 p.s.i. for 2 min. The pellets were resuspended and incubated for 90 min in 30 μ l of buffer (100 mM NaCl, 10 mM Na-phosphate pH 7.4, 1 mM EDTA) with α -BTX [30 μ g ml⁻¹; (+)] or without (-). Buffer was then added to 175 μ l and the samples were centrifuged as before. The final pellets were each resuspended in 20 μ l of H₂O. For the (+) sample (pre-incubated with α -BTX) no binding of ³H-labelled α -BTX was detected, indicating saturation of the available binding sites, whereas the value for the (-) sample was 1.4 nmol

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per mg protein. Negatively-stained electron microscope specimens were prepared in parallel, with the same batch of carbon film as reported elsewhere^{5,8}.

Figure 1a,b shows average images of membrane-bound AChR. They were obtained after alignment of individual images of negatively-stained specimens of AChR-rich membrane fragments (microsacs), from which non-receptor peptides had been removed by alkaline washing⁹. These microsacs had been incubated either with [Fig. 1a, (+)] or without [Fig. 1b, (-)] α -BTX. Figure 1a,b represents average axial projections (perpendicular to the membrane plane) of the stain-excluding mass in mem-

brane bound AChR. Their difference map [$\Delta = (+) - (-)$; Fig. 1c] shows two statistically significant peaks within the stain excluding region of the particle. Given that only about half the mass of each AChR molecule contributes to the stain exclusion⁶, we may roughly estimate from the size of the difference peaks that the mass added to either of the two regions on binding of α -BTX amounts to less than about one-quarter of the mass of an α -subunit (M_r 40,000).

Extensive tests were done to assess the reliability of the results. As the optical density $\bar{d}_{ik}$ in any image element (ik ; $i, k = 1, \dots, m$; $m = 32$) of an average image is the mean

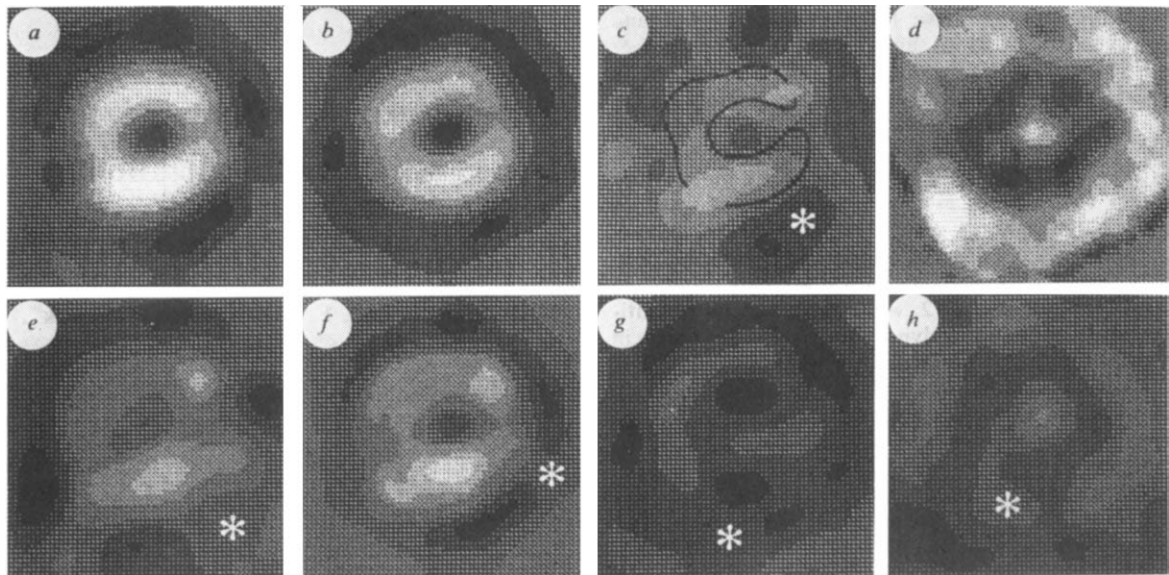


Fig. 1 Average images from negatively stained, membrane-bound AChR particles: *a*, obtained after incubation with α -BTX (+). *b*, Control [without α -BTX, (-)]. *c*, Difference [$\Delta = (+) - (-)$]. Optical density (OD) is low in bright and high in dark regions: thus, the stain-excluding mass appears bright. The density maximum of both *a* and *b* (in OD units) is 1.36. The grey levels in *a*, *b* and *c* are spaced at intervals of 0.029 OD units. Width of each map; 128 Å. *a*, *b* and *c* were low-pass-filtered to a resolution of 18 Å. For technical reasons related to image rotations and translations during alignment, only the central regions (~100 Å in diameter), but not the corners of the maps should be interpreted. The averages show the typical horseshoe-shape of the axial projections of negatively stained membrane-bound AChR particles^{5,6,21-23}. There are two regions where the stain-excluding mass increases on binding of α -BTX (*a*). This is clearly revealed by the difference map (*c*) which possesses two distinct negative peaks (-0.08 OD units) within the particle outline. The grey level closest to zero is marked by an asterisk. *d*, An unfiltered map of the standard error of the difference (s_{ik}^{Δ} , see text) using eight grey levels spaced at intervals of one-tenth of those in *a-c*. (Low values dark, high values bright, minimum 0.017, maximum 0.031.) The lowest errors occur within the region of the stain-excluding mass of the AChR particle. Computer processing (using the SPIDER software system²⁴) of digitized micrographs taken with a scanning transmission electron microscope (STEM) was as follows^{5,6}: a large field of view was segmented into small areas, each containing a single AChR particle. A circular mask (64 Å diameter) was used to screen off the surroundings of the particles^{5,6}. The rotation angles and translation vectors relative to a reference particle were determined by correlation functions, and the individual particles aligned and averaged. Each data set [(+) and (-)] comprised 40 particles. A particle chosen at random from the (+) set was used as a common reference for both data sets. In a second cycle (refinement) each set was aligned again, its previously obtained average now acting as the reference. The masked-off regions were aligned passively and included in the final average. For imaging in the STEM the elastically scattered electrons were used ('incoherent darkfield imaging'). With this type of instrument the image recording conditions (magnification, focus, minimized electron irradiation, optical density) are the same for each set of micrographs. Moreover, images were recorded within a few hours on the same strip of film, making scaling to compensate for instrumental variations unnecessary; that is, (for symbols see text) if the scaling procedure is described by $\bar{d}_{ik} = a\bar{d}_{ik} + b$, then $a = 1.0$, $b = 0$. This was confirmed by measurements of the optical density on unstained parts of the carbon films. There remained the possibility that either the two preparations were not equally embedded in negative stain or the stain was not equally dense. The respective optical density characteristics (maximum, minimum, mean, s.d.), as averaged over each of the two sets of micrographs, differed by ~20%. Assuming that this did not indicate structural differences, but unequal staining, the characteristics could be closely matched by setting $a = 1.2$. Thus, it became necessary to establish the effect that scaling might have had on the difference maps. (No scaling was applied to the data shown here.) If the mean density $\bar{\Delta}$ of the difference map is given by

$$\bar{\Delta} = (1/m^2) \sum_{i,k} \Delta_{ik}^+ \quad (\text{see text also})$$

and the image variance s_{Δ}^2 of the difference image by

$$s_{\Delta}^2 = \frac{\sum_{i,k} (\bar{\Delta} - \Delta_{ik}^+)^2}{m^2(m^2 - 1)}$$

we may take s_{Δ}^2 as a measure of the total amount of contrast variation (power) in the difference image. The effect of scaling on s_{Δ}^2 was investigated by varying a ($0.5 \leq a \leq 2.0$) in increments of 0.1. This indicated that the overall difference between the (+) and (-) averages within the stain-excluding region (surroundings screened off) can be minimized by setting $a = 1.3$. Nevertheless, for $0.8 \leq a \leq 1.5$, the height of the difference peaks (*c*) exceeded three times the standard error, s_{ik}^{Δ} of the difference ($3s_{ik}^{\Delta}$ -criterion; see *d* and text). On increasing a from 1.0 to 1.5 the difference peak at the lower left (designated α_1 in Fig. 3) moved radially by 10 Å from its position within the main stain-excluding region to a more peripheral position. The position of the other peak (α_2 in Fig. 3) was insensitive to scaling. No additional peaks emerged. These tests show that by applying physically reasonable scaling factors, the difference peaks cannot be made to disappear below the noise level and that the difference indeed indicates a net total increase in stain-excluding matter in the (+)-image relative to the (-)-image. The effect of optical density scaling was also investigated using the phase residual (see Fig. 2) as a criterion. The results were in full agreement with those discussed above. The difference maps *e-h* were obtained in the same manner (including the investigation of scaling effects) as described for *c* and are presented using the same density increment (0.029 OD units). Again, the asterisks mark the levels closest to zero; *e* and *f* are formed between the (+)-average and averages from untreated (O) or disulphide-reduced (R; using 10 mM dithiothreitol¹⁰) membrane fragments, respectively. Thus, *e* corresponds to (+)-(O) and *f* to (+)-(R). The density minima of *e* and *f* within the stain-excluding region are -0.085 and -0.11 OD units, respectively. The reproducibility of the averages may be assessed from the difference maps *g* and *h* which correspond to (-)-(-') and (-)-(R), respectively. Within the stain-excluding regions *g* and *h* show variations of $< \pm 0.03$ OD units, which are not significant according to the $3s_{ik}^{\Delta}$ -criterion applied throughout this work. In all difference maps, the peaks occurring in the stain regions would have to exceed ~0.1 OD units to be considered statistically significant. Note that the projection maps of the receptor particles differ from those presented earlier⁵. This is due not only to the application of a refinement cycle, but also to the inclusion of all particles in the course of the so-called '180°-decision'¹⁵. This applies to all data used here and is an improvement on our previous procedure, done in response to criticism raised by A. Brisson²¹, who independently confirmed the structure of the stain-excluding region by a different single-particle alignment method.

f N realizations d_{ik}^n ($n = 1, \dots, N$; $N = 40$)

$$\bar{d}_{ik} = (1/N) \sum_{n=1}^N d_{ik}^n$$

he standard error of the mean

$$s_{ik} = \sqrt{\frac{\sum_n (d_{ik}^n - \bar{d}_{ik})^2}{N(N-1)}}$$

was used as a criterion for assessing the significance of the difference $\Delta_{ik}^+ = \bar{d}_{ik}^+ - \bar{d}_{ik}^-$ (Fig. 1a,b) between corresponding image elements in the averages of the two sets. This difference was considered significant if it exceeded three times the standard errors (s_{ik}^+ , s_{ik}^-) of the (+)– and (–)–averages. A map of the standard error of the difference

$$s_{ik}^\Delta = \sqrt{(s_{ik}^+)^2 + (s_{ik}^-)^2}$$

is shown in Fig. 1d. To apply this criterion we must assume that the values of d_{ik}^n have a gaussian distribution, and that the images are aligned correctly (see Fig. 1 legend). The hypothesis that the d_{ik}^n are normally distributed could not be rejected (Kolmogorov–Smirnov test¹⁰). The reliability of the alignment procedure can be deduced from a comparison of independent averages, using the phase residual as a measure of the reproducible resolution¹¹ (Fig. 2). Additionally, a non-crystallographic test¹² was developed on the basis of non-parametric statistical methods¹³ and applied to aligned and non-aligned image sets. This test requires neither gaussian distributions nor statistical independence of the data; for any image element of an average image and at any significance level it specifies a threshold to be exceeded by the contrast of nearby elements. This method established that after alignment, the density of the stain-excluding regions varied significantly (at the 95% confidence level) over distances of ~ 20 Å. When the images had been aligned merely by translation, omitting rotational alignment (so

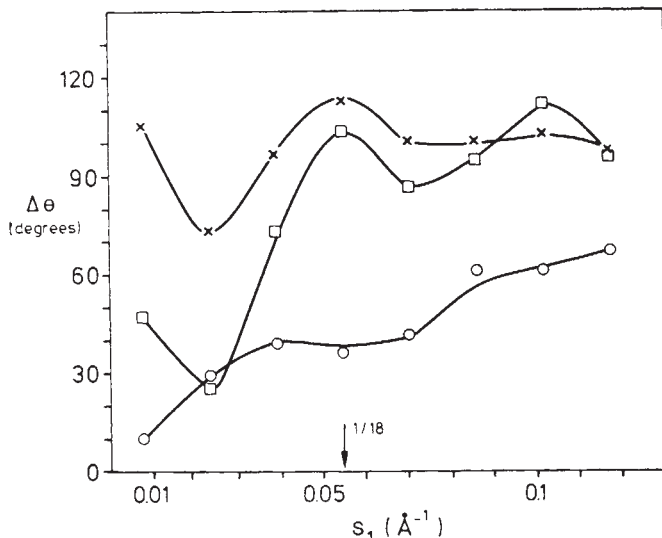


Fig. 2 Differential plots of the phase residual¹¹

$$\Delta\theta = \sqrt{\frac{(|F_1| + |F_2|)\Delta\delta^2}{(|F_1| + |F_2|)}}$$

where F_1 and F_2 denote corresponding complex Fourier coefficients of two (independent) average images and $\Delta\delta$ is their phase difference. The summations extend over a ring zone $S_1 \pm \Delta S$ of the reciprocal space. This analysis shows that $\Delta\theta$ is less than 45° when independent averages (○) taken over >20 aligned images are compared, and that presentation at 18 Å resolution is justified. The plots also illustrate the improvement achieved on alignment by comparing two arbitrarily chosen single particles before (×) and after (□) alignment. In this analysis, which was aimed at demonstrating the reproducible resolution in the stain-excluding mass of the AChR particles, a circular mask (64 Å diameter) was used to screen off the particle surroundings.

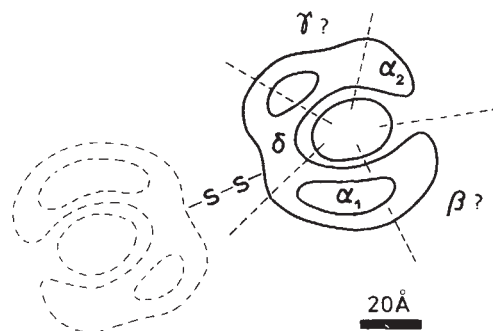


Fig. 3 Schematic representation of the subunit topography of the membrane-bound AChR protein viewed in axial projection perpendicular to the membrane plane. The approximate positions of the α_1 -, α_2 - and δ -chains have been determined directly.

that only their stain-filled centres were in register), significant density variations were lacking in the diffuse annulus representing the average stain-excluding mass.

In the preparation of the specimens, we applied an alkaline washing step⁹ to improve the image contrast of the AChR particles⁸. It extracts non-receptor peptides without either affecting the function (α -BTX binding, ion fluxes) of the AChR^{8,14} or altering the lipid composition of the microsacs¹⁵. Extraction of non-receptor peptides does not affect the internal structure of the stain-excluding mass of membrane-bound AChR¹⁶. Changes occur only near the periphery of the particles (unpublished results); they differ from the effect of disulphide reduction which abolishes a pairwise association of nearest-neighbour particles⁶. Thus, difference maps may also be formed between the average shown in Fig. 1a and averages from untreated microsacs (○) or from microsacs after disulphide reduction (R). These maps ((+)–(○); Fig. 1e, and (+)–(R); Fig. 1f) show essentially the same features as (+)–(–) (Fig. 1c); phase residuals between these maps were consistently smaller than 45° for spatial frequencies $< 1/18$ Å^{–1}. Accordingly, difference maps between the averages without α -BTX in various combinations, for example, between (–) and a corresponding average (–') from an independent data set [(–)–(–')] (Fig. 1g; see also Fig. 2) or (–)–(R) (Fig. 1h) lacked significant peaks within the stain-excluding region.

We therefore believe that the two difference peaks in Fig. 1c are not only statistically significant, but indeed are due to bound α -BTX marking the α -subunits (α_1 , α_2 ; Fig. 3). A substantial exclusion of stain by the toxin might be expected because the 'long toxins' are known to have a rather compact structure stabilized by five disulphide bridges^{3,17}. Furthermore, a compact AChR–toxin complex is suggested by the extensive surface contacts between toxin and AChR (~ 200 van der Waals contacts have been estimated¹⁸). Nevertheless, the unequal sharpness of the two difference peaks indicates that the precision of the localization is not the same for α_1 and α_2 .

Although this study provides no clues as to the location of the putative ACh-activated ion channel, it excludes the possibility that α -BTX acts by physically blocking the entrance to the central cavity of the hydrated mass in the membrane-bound AChR. From the available three-dimensional data^{6,16}, we estimate that this cavity—visualized by the densely stained pit—could accommodate a protein mass of $M_r \sim 10,000$. α -BTX (M_r 8,000) bound to this region would have been clearly revealed.

In Fig. 3 we have also indicated the previously obtained position of the δ -subunit as defined by the contact region between adjacent AChR monomers that are disulphide-linked between their respective δ -subunits⁶. As the δ – δ bond is well documented², the δ -subunit is a convenient reference point for establishing the relative topography of other subunits. The positions of $\beta?$ and $\gamma?$ in Fig. 3 are based merely on the following circumstantial evidence. Extraction of non-receptor peptides (mainly of the M_r 43,000 component (ν)⁹ by alkaline

washing uncovers antigenic determinants on α , γ and δ (ref. 14). Depletion of skin-excluding mass on alkaline washing was observed (unpublished results) in the neighbourhood of the AChR, near α_2 and δ . This led to the tentative assignment of γ to the area between α_2 and δ (Fig. 3).

Thus, we conclude that one of the two α -chains (α_1) is situated in the immediate vicinity of a δ -chain, and that α_2 is ~ 50 Å away. Biochemical labelling and cross-linking studies (reviewed by Karlin² and Hucho⁷) have shown that all chains of the AChR are in close proximity. Despite considerable difficulties in their interpretation, such experiments provide evidence that α and δ are the species most readily cross-linked to one another, whereas cross-links between the two α -chains have not been found. Our result, while in broad agreement with these conclusions, helps to resolve the fundamental difficulty that cross-

linking experiments give equivocal results if they cannot distinguish between the two copies of the α -chain. In membrane bound AChR the two recognition sites, one on each α -chain are indistinguishable in their ability to bind snake α -toxins, and in the kinetics of this binding¹⁹. On the other hand, difference seem to exist with respect to affinity labelling^{2,20}. A further difference is directly manifested in the environment of each α -chain (Fig. 3): each toxin-binding region exhibits contact with a different set of neighbouring subunits, reinforcing the evidence for the asymmetric quaternary structure of the AChR-protein.

We thank Jan de Maeyer and Angelo di Nicola for system support related to computing. This work was funded by Deutsche Forschungsgemeinschaft grants DFG Zi 224/1 and DFG Zi 224/2-1. Reprint requests should be addressed to F.J.B.

Received 31 December 1981; accepted 5 July 1982.

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The linear Onsager coefficients for biochemical kinetic diagrams as equilibrium one-way cycle fluxes

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In a biochemical kinetic diagram¹, the various discrete states of an enzyme or enzyme complex are represented by points, and interstate transitions are represented by lines. Figure 1 is an example. I show here that, for steady states near equilibrium, the phenomenological Onsager coefficients in the linear flux-force equations that are a consequence of any such diagram have a transparent physical interpretation: these coefficients are simple combinations of one-way cycle fluxes¹ at equilibrium. The existence of this kind of relationship was pointed out recently in an appendix of a paper² on another subject. I present here the explicit connection between the phenomenological coefficients and the one-way cycle fluxes of the diagram. These coefficients are considered by many scientists to be strictly empirical and incomprehensible. My report provides a simple interpretation of them, at the molecular level, for any biochemical kinetic diagram. This is analogous to the molecular interpretation of phenomenological equilibrium thermodynamic quantities that is provided by statistical mechanics.

Instead of treating a completely arbitrary diagram with generalized notation, the general rules can easily be deduced from a special case that has sufficient complexity. For this purpose we use the hypothetical model of the (Na⁺ + K⁺)ATPase complex shown in Fig. 1. First, cycles and one-way cycle fluxes are introduced for this model, and then their relationship to the Onsager linear coefficients is pointed out.

The hypothetical diagram (or reaction mechanism) in Fig. 1a has eight enzyme states and six cycles. The latter are shown in Fig. 1b. The anticlockwise direction is chosen as positive. The

dominant cycle is 'g'. The enzyme complex is in a membrane separating 'in' and 'out'. For each completed g cycle, in the +direction, two K⁺ ions are transported out $\rightarrow$ in, three Na⁺ ions are transported in $\rightarrow$ out and one ATP molecule is hydrolysed. The transitions 2 $\rightleftharpoons$ 7 and 3 $\rightleftharpoons$ 6 introduce some slippage and also introduce the other five cycles included in Fig. 1b: cycle a (+ direction) transports two K⁺ out $\rightarrow$ in, cycle d (+ direction) transports two K⁺ out $\rightarrow$ in and three Na⁺ in $\rightarrow$ out, and so on.

The observable steady-state fluxes, per complex, are denoted J_K , J_N and J_T , for K⁺, Na⁺ and ATP, respectively. These are all chosen as positive in the normal operating directions specified above. The corresponding thermodynamic forces, per ion or molecule, are then X_K (out $\rightarrow$ in), X_N (in $\rightarrow$ out) and X_T , where X_K and X_N are negative, and X_T is positive. In normal operation, K⁺ and Na⁺ are driven across the membrane against their thermodynamic forces by the dominant force X_T .

There are 10 lines in the diagram and hence 20 first-order (or pseudo-first-order) rate constants (units, say, s⁻¹). If these rate constants are all specified, the three observable steady-state fluxes can be written as sums of separate cycle contributions¹:

$$\begin{aligned} J_K &= 2(J_a + J_d + J_g) \\ J_N &= 3(J_b + J_d + J_f + J_g) \\ J_T &= J_c + J_f + J_g \end{aligned} \quad (1)$$

All these fluxes are in units of s⁻¹ per complex. J_a is the net mean rate of cycle a completions in the +direction, and so on. Cycles a, d and g appear in the J_K equation because (see Fig. 1) these and only these three cycles are involved in K⁺ transport (two K⁺ per cycle). Each cycle flux J_κ ($\kappa = a, \dots, g$) above can be expressed in terms of the first-order rate constants of the diagram in the form¹

$$J_\kappa = (\Pi_{\kappa^+} - \Pi_{\kappa^-})F_\kappa \quad (2)$$

where Π_{κ^+} is the product of rate constants around cycle κ in the positive direction, Π_{κ^-} is the same for the negative direction and F_κ is a positive algebraic combination of all 20 rate constants of the diagram (the details¹ of which are not needed here).

Actually, κ cycles can be completed in either direction, and J_κ can be decomposed as follows¹:

$$J_\kappa = J_{\kappa^+} - J_{\kappa^-}, \quad J_{\kappa^+} = \Pi_{\kappa^+} F_\kappa, \quad J_{\kappa^-} = \Pi_{\kappa^-} F_\kappa, \quad (3)$$

where J_{κ^+} is the mean rate (s^{-1}) at which κ cycles are completed in the + direction at steady state, and similarly for J_{κ^-} . The fluxes J_{κ^+} and J_{κ^-} are the one-way cycle fluxes for cycle κ . Both J_{κ^+} and J_{κ^-} are positive but J_κ might be negative. Separate cycle fluxes or separate one-way cycle fluxes are generally not directly observable. If a tracer were used in our example, one could observe, say, the sum

$$J_{\kappa^+} = 2(J_{a^+} + J_{d^+} + J_{g^+}) \quad (4)$$

but not J_{a^+} , J_{d^+} and J_{g^+} separately. However, as will be seen below, it is possible in some cases to determine, experimentally, equilibrium values of one-way cycle fluxes. Returning to equation (3), we note that

$$J_{\kappa^+}/J_{\kappa^-} = \Pi_{\kappa^+}/\Pi_{\kappa^-} \quad (5)$$

in which F_κ does not appear.

The thermodynamic force X_κ , operating in cycle κ , is determined by the processes included in cycle κ :

$$X_a = 2X_K, \quad X_b = 3X_N, \quad X_c = X_T, \quad X_d = 2X_K + 3X_N \quad (6)$$

$$X_f = 3X_N + X_T, \quad X_g = 2X_K + 3X_N + X_T$$

Each cycle force X_κ is simply related¹ to Π_{κ^+} and Π_{κ^-} for the cycle:

$$\Pi_{\kappa^+}/\Pi_{\kappa^-} = e^{X_\kappa/kT} = J_{\kappa^+}/J_{\kappa^-} \quad (7)$$

where the last relation makes use of equation (5).

The above discussion applies to an arbitrary steady state. Here, steady states near equilibrium are considered. For this we require

$$|X_K|/kT, \quad |X_N|/kT, \quad |X_T|/kT \ll 1 \quad (8)$$

However, if only cycle g is important, the near-equilibrium condition is much less stringent:

$$|X_g|/kT = |2X_K + 3X_N + X_T|/kT \ll 1 \quad (9)$$

Only the sum need be small, not the separate forces. This important special case will be referred to again below.

Returning to the general case, equation (8), remember that the model in Fig. 1 is being used here simply as a sufficiently complicated example from which to see general results. The conditions in equation (8), especially $|X_T|/kT \ll 1$, are certainly not important for the real ($\text{Na}^+ + \text{K}^+$)ATPase (see below in this connection). Near equilibrium, the phenomenological flux-force relations are, as is well known,

$$J_K = L_{KK}X_K + L_{KN}X_N + L_{KT}X_T$$

$$J_N = L_{NK}X_K + L_{NN}X_N + L_{NT}X_T \quad (10)$$

$$J_T = L_{TK}X_K + L_{TN}X_N + L_{TT}X_T$$

where, as Onsager showed for a wide class of systems, the matrix L_{ij} is symmetric (that is, $L_{ij} = L_{ji}$). Our object is to relate the L_{ij} for a biochemical kinetic diagram to the one-way cycle fluxes. In doing this, the symmetry of the matrix will appear automatically.

At an arbitrary steady state, the rate of free energy dissipation is

$$Td_iS/dt = J_KX_K + J_NX_N + J_TX_T \geq 0 \quad (11)$$

Here X_K and X_N are negative and the other four factors are positive. Near equilibrium, using equations (10) and $L_{ij} = L_{ji}$,

$$Td_iS/dt = L_{KK}X_K^2 + L_{NN}X_N^2 + L_{TT}X_T^2 + 2L_{KN}X_KX_N + 2L_{KT}X_KX_T + 2L_{NT}X_NX_T \geq 0 \quad (12)$$

This is a non-diagonal quadratic form in which the last two terms are usually negative.

We turn now to a consideration of cycles. In view of equations (6) and (8), all of the $|X_\kappa|/kT \ll 1$. From equations (3) and (7),

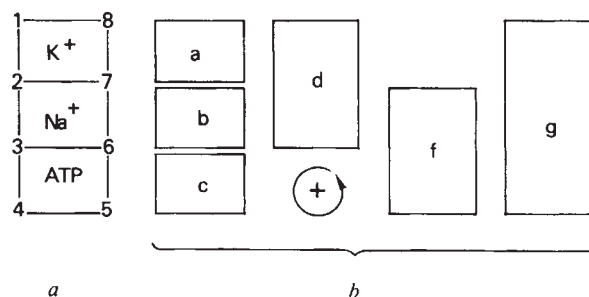


Fig. 1 Hypothetical model of ($\text{Na}^+ + \text{K}^+$)ATPase in a membrane, in the form of a biochemical kinetic diagram (a). The enzyme complex has eight significant states, which need not be specified explicitly. The diagram has six cycles (b). All fluxes are chosen as positive in the anticlockwise direction.

we have then

$$J_\kappa = J_{\kappa^+} - J_{\kappa^-} = J_{\kappa^+}[(J_{\kappa^+}/J_{\kappa^-}) - 1]$$

$$= J_{\kappa^+}(e^{X_\kappa/kT} - 1) = J_{\kappa^+}^\circ(X_\kappa/kT) + \dots \quad (13)$$

The last form is the near-equilibrium special case. At equilibrium, the two one-way cycle fluxes for cycle κ are equal to each other, because $J_\kappa = 0$. That is, $J_{\kappa^+}^\circ = J_{\kappa^-}^\circ$. We denote either of these by $J_{\kappa^\pm}^\circ$ [as in equation (13)]. $J_{\kappa^\pm}^\circ$ is a positive quantity; it is the one-way cycle flux, at equilibrium. $J_{\kappa^\pm}^\circ$ can be expressed¹ in terms of the rate constant set of the diagram at equilibrium, using equation (3), but the physical interpretation of $J_{\kappa^\pm}^\circ$, just mentioned, is the main point of interest here.

If we now substitute the last form of equation (13), for cycles a , d and g , into the J_K equation in equations (1), and then use equations (6) to eliminate X_a , X_d and X_g , we obtain

$$J_K = 2\left[J_a^\circ\left(\frac{2X_K}{kT}\right) + J_d^\circ\left(\frac{2X_K}{kT} + \frac{3X_N}{kT}\right) + J_g^\circ\left(\frac{2X_K}{kT} + \frac{3X_N}{kT} + \frac{X_T}{kT}\right)\right]$$

$$= 4(J_a^\circ + J_d^\circ + J_g^\circ)(X_K/kT) + 6(J_d^\circ + J_g^\circ)(X_N/kT) + 2J_g^\circ(X_T/kT) \quad (14)$$

If we follow the same procedure for J_N and J_T , and then compare with equations (10), we find for the L_{ij} :

$$L_{KK} = 4(J_a^\circ + J_d^\circ + J_g^\circ)/kT$$

$$L_{NN} = 9(J_b^\circ + J_e^\circ + J_f^\circ + J_g^\circ)/kT$$

$$L_{TT} = 1(J_c^\circ + J_f^\circ + J_g^\circ)/kT \quad (15)$$

$$L_{KN} = L_{NK} = 6(J_d^\circ + J_g^\circ)/kT$$

$$L_{KT} = L_{TK} = 2J_g^\circ/kT$$

$$L_{NT} = L_{TN} = 3(J_f^\circ + J_g^\circ)/kT$$

The symmetry of the matrix appears at this point. The rules for the construction of equations (15), merely by inspection of the diagram and cycles, are obvious from the first form of equation (14): (1) those cycles that include process i contribute to L_{ii} ; (2) those cycles that include both process i and process j contribute to L_{ij} (hence the matrix is symmetrical); (3) the integral numerical coefficients in the L_{ij} are determined by a matrix of stoichiometric products. In the present example, the product matrix is (order K, N, T).

$$\begin{pmatrix} 2.2 & 2.3 & 2.1 \\ 3.2 & 3.3 & 3.1 \\ 1.2 & 1.3 & 1.1 \end{pmatrix} \quad (16)$$

From the simple construction of the first form of equation (14), it is clear that the above rules apply to any diagram. Thus there is a simple physical interpretation of the L_{ij} matrix for any diagram in terms of the equilibrium one-way cycle fluxes of the cycles of the diagram.

The signs of the L_{ij} ($i \neq j$) need not always be positive, as in this example. Thus, suppose we reverse the signs of J_K and X_K in equations (1) and (6) (this is a matter of convention), then we find that a minus sign appears in the expressions for $L_{KN} = L_{NK}$ and for $L_{KT} = L_{TK}$ in equations (15). This was, in fact, the choice made in ref. 1, equation (3.66). However, negative signs can be avoided by choosing all fluxes (operational and cycle) as positive in the same direction.

In principle, the L_{ij} can all be measured by observing the operational steady-state fluxes and forces in equations (10), near equilibrium. With these available, all of the J_K^e can be deduced from equations (15), in the order, for example, g, d, f, a, c, b. Thus, the equilibrium one-way cycle fluxes can be obtained from conventional flux-force measurements. This will be possible, in principle, whenever the number of cycles (with non-zero force and flux¹) in the diagram does not exceed $n(n+1)/2$ (the number of independent L_{ij}), where n is the number of operational fluxes (or forces) in the system.

If only cycle g contributes to the fluxes, we have, near equilibrium

$$J_K = 2J_g^e (X_g/kT), \quad J_N = 3J_g^e (X_g/kT), \quad J_T = J_g^e (X_g/kT) \quad (17)$$

where X_g is given in equation (6). Each linear coefficient is determined by the equilibrium one-way cycle flux of the only operative cycle and by the stoichiometry of that cycle. Here we have integral 2:3:1 stoichiometry between the observable fluxes. If other cycles participate, this exact stoichiometry will be lost.

If we substitute equations (1) into equation (11), and then use equations (6), the rate of free energy dissipation at an arbitrary steady state can be expressed in terms of cycle contributions rather than of observable process contributions:

$$T d_i S/dt = J_a X_a + J_b X_b + J_c X_c + J_d X_d + J_f X_f + J_g X_g \geq 0 \quad (18)$$

Whereas $J_K X_K$ and $J_N X_N$ in equation (11) are negative, no term in equation (18) can be negative. This follows from equations (2) and (7): J_K and X_K have the same sign. Near equilibrium, we put the last form of equation (13) into equation (18), in place of each J_K , and obtain

$$(1/k) d_i S/dt = J_a^e (X_a/kT)^2 + \dots + J_g^e (X_g/kT)^2 \geq 0 \quad (19)$$

Unlike equation (12), this is a diagonalized quadratic form, with every term positive.

Equations (18) and (19), for cycles, have the same mathematical properties as when the individual molecular transitions of a diagram are used (see, for example, equation (4.28) of ref. 1). That is, if we advance, conceptually, from the molecular level to the cycle level these properties are still retained, though they are lost if we go still further to the observable process level, as mentioned following equations (11) and (12). Thus, although cycles are composites of many molecular transitions, in some thermokinetic respects they still resemble individual transitions (for further discussion, see ref. 1).

Although the analogy to the present cycles is not close, it is interesting that Haase³ has shown that the L_{ij} for an arbitrary set of coupled homogeneous chemical reactions can be expressed in terms of one-way reaction fluxes at equilibrium.

We are here primarily concerned with the general question of the relationship between the L_{ij} and cycles for an arbitrary diagram, near equilibrium. Essentially as an addendum we note that, as is well known, many free energy transducing systems in membranes seem to be dominated by a single cycle (for example, cycle g in Fig. 1b) that operates rather near to equilibrium; other slippage cycles are probably used to a limited extent but the forces in these cycles are very far from equilibrium. Thus, if more than one (the main) cycle is used, the above uniformly-near-equilibrium treatment does not apply.

The model in Fig. 1 for $(Na^+ + K^+)ATPase$ can be used as an illustration. In normal operation, X_K and X_N are large and negative, X_T is large and positive, but $X_g = 2X_K + 3X_N + X_T$ is small and positive (cycle g is near equilibrium). Consequently,

from equations (6),

$$\begin{aligned} X_a, J_a; X_b, J_b; X_d, J_d & \text{ are negative} \\ X_c, J_c; X_f, J_f & \text{ are positive} \end{aligned} \quad (20)$$

The forces in expression (20) are all large in magnitude but the fluxes are relatively small because the slippage rate constants ($2 \rightleftharpoons 7$, $3 \rightleftharpoons 6$) are relatively small. Furthermore, these cycle fluxes will be essentially one-way cycle fluxes, because of the large cycle forces [equation (7)]. Because X_g is positive, J_g is positive. Equations (1), in this case, become

$$\begin{aligned} J_K &= 2[-J_a - J_d + J_g^e (X_g/kT)] \\ J_N &= 3[-J_b - J_d + J_f + J_g^e (X_g/kT)] \\ J_T &= J_c + J_f + J_g^e (X_g/kT) \end{aligned} \quad (21)$$

The one-way cycle fluxes $J_a^e, \dots, J_f^e$ that appear here are not equilibrium fluxes. The corresponding cycle forces do not enter these equations because, for these cycles, the activity is almost one-way (irreversible). The cycle g terms usually dominate; the other terms are slippage corrections which, incidentally, would spoil the exact 2:3:1 stoichiometry of cycle g. All the terms in equations (21) can be expressed¹ as functions of the rate constants of the diagram at this steady state.

I thank Dr S. R. Caplan for significant suggestions and corrections.

Received 9 March; accepted 15 June 1982.

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ATP-dependent unwinding of double helix in closed circular DNA by RecA protein of *E. coli*

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RecA protein is an essential component directly involved in general genetic recombination in *Escherichia coli*^{1,2}. In the presence of ATP, RecA protein promotes *in vitro* pairing of homologous DNA molecules^{3–9}, unidirectional elongation of heteroduplex joints^{7,10–12} and concerted reciprocal strand exchange^{13,14}. These activities of the protein can explain some important steps in genetic recombination (see ref. 15 for review). When negative-superhelical closed-circular double-stranded DNA (form I DNA) is a substrate, RecA protein catalyses a cycle of sequential reactions: formation and dissociation of D-loops, and inactivation and reactivation of the duplex as a substrate for D-loop formation^{16,55}. We now present evidence that, in the presence of ATP, RecA protein unwinds the double helix of form I DNA to produce positive-superhelical turns which can be relaxed by eukaryotic topoisomerase I and that, by this process, closed-circular double-stranded DNA with an extraordinarily large number of negative-superhelical turns can be formed in physiological conditions. This ATP-dependent unwinding of the double-helix might promote dissociation of D-loops in form I DNA, followed by inactivation of the form I DNA and the elongation of heteroduplex joints, as suggested by our previous studies^{16,17,55}.

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The superhelicity of DNA is thought to play a part in genetic recombination^{15,18-22}. When form I DNA was incubated with homologous single-stranded fragments, excess ATP and excess RecA protein at 37 °C, D-loops were formed in the early stage of the incubation and then completely dissociated^{16,17,55} (Fig. 2a). When D-loops are completely dissociated, all form I DNA becomes an inactive substrate for D-loop formation¹⁶. These latter two processes can be explained by a model in which free RecA protein (RecA protein that does not bind to single-stranded DNA) binds to double-stranded DNA cooperatively from the site of the D-loop and unwinds the double helix unidirectionally. Unidirectional unwinding of the double helix in form I DNA in the absence of any topoisomerase activity^{23,24} results in relaxation of negative supercoiling and then the accumulation of positive supercoiling, followed by rewinding of the double helix at the trailing end of the unwound region to relax the positive supercoiling (illustrated in Fig. 1, steps from (i) to (iii) via (ii)).^{16,17,55}

To obtain direct evidence for unwinding of the double-helix in form I DNA in an inactivated state, we performed the following experiments. After the formation and subsequent complete dissociation of the D-loops by incubation of form I ³H-labelled DNA with homologous single-stranded fragments, ATP and RecA protein for 50 min (the first incubation), we treated the form I DNA for 5 min at 37 °C with an eukaryotic topoisomerase I prepared from *Saccharomyces cerevisiae* (the second incubation). Eukaryotic topoisomerase I, including that from *S. cerevisiae*, is able to relax both positive and negative supercoiling (see refs 25, 26 for review, also H.W. and T.I., unpublished observation). When both the first incubation and RecA protein in the second incubation were omitted, the duplex DNA was completely converted to relaxed form (form IV) by the second incubation and exhibited a set of closely spaced bands near a band produced by nicked-circular DNA (form II

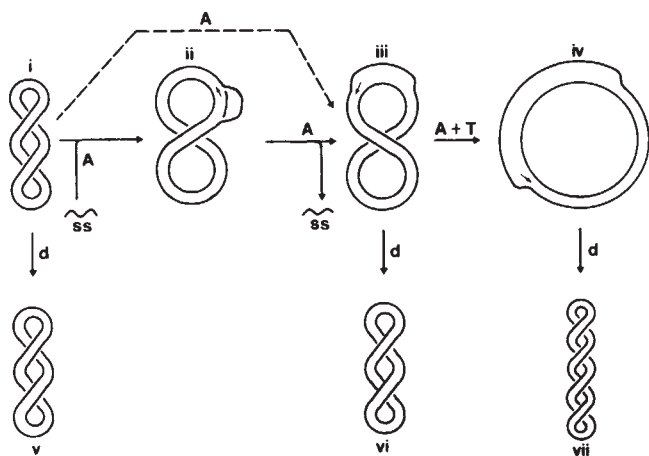


Fig. 1 Schematic diagram of the experiments to demonstrate unwinding of the double helix by RecA protein. Basically, when we compare the average linking numbers between a closed-circular DNA relaxed by topoisomerase in the presence of double helix unwinding reagents (iv) or (vii), and that relaxed in their absence, the difference directly indicates the angle of unwinding by the reagents⁴⁹⁻⁵¹. The double helix is unidirectionally unwound by RecA protein (indicated by small arrows in (ii), (iii) and (iv)). Unidirectional unwinding by RecA protein is initiated very efficiently at the site of a D-loop (small arrow in (ii)). In the case of negative-superhelical DNA (form I DNA), the initiation occurs at a very low rate even in the absence of any single-stranded DNA (indicated by broken line from (i) to (iii)), probably at transient denatured sites. Because, in the absence of topoisomerase activity, the unidirectional unwinding of the double-helix in closed-circular DNA results in the loss of negative supercoiling of form I DNA, dissociation of D-loops (steps from (ii) to (iii)) and then positive supercoiling of the form I DNA (iii). The product is then treated with eukaryotic topoisomerase I in the presence of active RecA protein (indicated by A+T). The positive supercoiling of the form I DNA (iii) is relaxed and the unwound region extended (iv). After proteins are removed (step indicated by d), product (iv) becomes a form (vii) with a larger number of negative-superhelical turns than natural form I DNA (v). When the double helix is not unwound, negative supercoiling of form I DNA is relaxed with topoisomerase I. A, T and ss denote RecA protein, topoisomerase I and homologous single-stranded fragments, respectively.

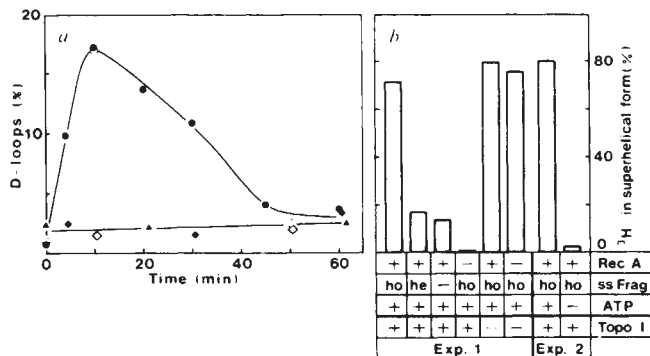


Fig. 2 Formation and dissociation of D-loops, and formation of form X. fd form I ³H DNA preparation (4 μM, containing 75% form I DNA) and 0.05 μM homologous single-stranded fragments were incubated with 1 μM (in a, open symbols, and in b) or 0.5 μM (in a, closed symbols) RecA protein (molecular weight 40,000) and 1.3 mM ATP in a reaction mixture (20.5 μl) containing 31 mM Tris-HCl (pH 7.5), 13 mM MgCl₂, 1.8 mM dithiothreitol and 88 μg of bovine serum albumin per ml at 37 °C (the first incubation). RecA protein is a DEAE-cellulose fraction (fraction V) prepared as described previously²⁴. Preparation of DNA is described elsewhere^{5,24}. Amounts of DNA are expressed in moles of nucleotide residues. a, Samples were withdrawn at the indicated times and D-loops were assayed by the nitrocellulose-filter method^{16,33}. ○, ●, Complete reaction mixture; ◇, ◆, with heterologous (ΦX174) fragments; △, ▲, without RecA protein. b, After treatment of fd form I DNA by the first incubation of 50 min, topoisomerase I from *S. cerevisiae*⁵² (30 μg ml⁻¹) was added to the reaction mixture, followed by incubation for 5 min at 37 °C. Reactions were terminated by chilling in an ice-water bath and by denaturing proteins with 0.5% Sarkosyl NL97 (Ciba-Geigy) and 4 μl of phenol saturated with the buffer. Samples were then electrophoresed through 1% agarose gel in E-buffer⁵³ in the absence of dye at 20 V for 15 h at room temperature. After electrophoresis, the agarose gel (shown in Fig. 3) containing each lane was cut into several pieces, dissolved by boiling for 2–4 min with 0.1 M HCl, applied to a glass filter (Whatman GFC) and dried. Radioactivity on the filter was assayed in a Beckman LS8100 scintillation counter by using toluene scintillator. Percentage of radioactivity in bands formed by form I DNA and form X DNA to total recovered radioactivity is plotted. Topoisomerase I, with a molecular weight of 70,000 as estimated by gel filtration was purified ~8,000-fold by a series of chromatographies on an Ultrogel AcA 34 column, a double-stranded DNA-cellulose column and a hydroxylapatite column from the phosphocellulose fraction of *S. cerevisiae* IAM4274 prepared as described previously⁵⁴. This preparation of the topoisomerase is free of detectable endo- or exo-DNase, ATPase or DNA ligase activities and relaxes both positive and negative supercoiling by changing linking number in steps of one (H. W. and T. I., unpublished observation). 'ho' and 'he' indicate homologous single-stranded fragments and heterologous ones, respectively. RecA, ss frag and topo I indicate RecA protein, single-stranded fragments and the second incubation with topoisomerase I, respectively.

DNA) in the gel electrophoregram (Fig. 3, lane 9); about 99% of the recovered radioactivity of the input duplex DNA was in the bands of form IV and form II DNA. However, a large part of the duplex DNA from the complete reaction system formed a band which migrated significantly faster than the untreated form I DNA (Fig. 3, lane 14 compared with lanes 1 and 17). Here we tentatively call the form of DNA migrating faster than natural form I DNA form X, for simplicity.

We concluded from the following observations that form X is a closed-circular double-stranded DNA with an extraordinarily large number of negative-superhelical turns or much smaller linking number than natural form I DNA. Ethidium bromide is an intercalating dye which unwinds the double helix²⁷⁻²⁹. As shown in Fig. 4b, in the presence of 0.15 μM ethidium bromide, untreated form I DNA behaved as the relaxed form in gel electrophoresis (lane 4), and form IV DNA as the positive-superhelical form (lanes 1–3). In this condition, form X DNA still behaved as a superhelical form (Fig. 4b, lanes 5–7). Form X DNA formed by the second incubation for 20 s could be relaxed by 0.7 μM ethidium bromide in the gel (Fig. 4c, lane 5). In the presence of this concentration of the dye, both form I and form IV DNA behaved as the positive-superhelical form (Fig. 4c, lanes 1–4). The form X DNA formed by the second incubation for 5 min and that formed by the second incubation for 15 min were not relaxed by this concentration

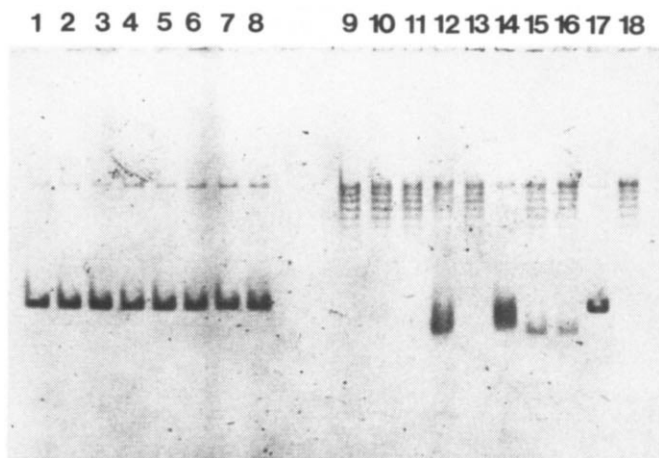


Fig. 3 Gel electrophoresis of the product formed by RecA protein and topoisomerase I. Conditions for the reaction are given in Fig. 2 legend. As the length of single-stranded fragments used in this study was about 200 nucleotides, the fd form I DNA bearing a D-loop which was formed with these fragments was not relaxed (lane 4). The band produced by the single-stranded fragments was not significant in the conditions used here, because the single-stranded fragments migrated in a broad band and their concentration was only 1/70th of the duplex DNA. For lanes 1–8, the second incubation was omitted. The period of the first incubation was as indicated. For lanes 9–16, reaction mixtures were incubated with topoisomerase I for 5 min at 37°C after the indicated period of the first incubation. Lanes 1 and 9, without RecA protein for 0 min; lanes 2 and 10, complete reaction mixture for 0 min; lanes 3 and 11, with heterologous single-stranded fragments for 0 min; lanes 4 and 12, complete reaction mixture for 10 min; lanes 5 and 13, with heterologous single-stranded fragments for 10 min; lanes 6 and 14, complete reaction mixture for 50 min; lanes 7 and 15, with heterologous single-stranded fragments for 50 min; lanes 8 and 16, without single-stranded fragments for 50 min; lane 17, untreated form I DNA; lane 18, after 50 min of the first incubation of complete reaction mixture, 3 mM ADP was added to the reaction mixture and incubated for 30 min at 37°C. Then topoisomerase I was added and incubated for 5 min at 37°C. After electrophoresis, we photographed the gel as described previously²⁴.

of ethidium bromide (Fig. 4c, lanes 6, 7); the former was relaxed by the dye at 1–3 μM and the latter by the dye at $>5 \mu\text{M}$. These experiments also indicate that the extent of unwinding of the double helix in the closed-circular DNA gradually increases during the second incubation. A preliminary quantitative measurement of superhelicity using the ethidium bromide–buoyant density technique³⁰ showed that the form X formed

by the second incubation for 20 s has about twofold more superhelical turns than natural form I DNA (T.O. and T.S., unpublished result). This amount of supercoiling corresponds to the unwinding of about 800 base pairs (see ref. 24). As the velocity of unwinding of the double helix in form I DNA by RecA protein was calculated to be about one base pair per second¹⁶, the above experiments suggest that during the first incubation for 50 min, positive-superhelical turns seem to be accumulated in the duplex DNA (Fig. 1, product (iii)).

The amount of topoisomerase I added in these experiments completely relaxed form I DNA in the absence of RecA protein within 20 s (Fig. 4a, lane 1). After the first incubation for 50 min, 20 s of the second incubation was enough to convert almost all of the closed-circular DNA to form X (Fig. 4a, lane 5); 74% of the recovered radioactivity of the input duplex DNA which contained 77% form I DNA was in the band of form X in the gel electrophoregram in the absence of ethidium bromide (Fig. 4a, lane 5), and 92 and 8% of the radioactivity were in the bands of relaxed form and in the band of unreacted form I DNA, respectively, in the presence of 0.7 μM ethidium bromide (Fig. 4c, lane 5).

The yield of form X DNA was the function of the period of the first incubation. When the first incubation was omitted, no form X was formed during the second incubation (Fig. 3, lane 10), and when the first incubation was not long enough to complete the dissociation of D-loops, a smaller portion of form I DNA was converted to form X (Fig. 3, compare lanes 12 and 14; see Fig. 2a). Therefore, the double helix of all form I DNA in an inactive state is extensively unwound and this unwinding correlates with the inactivation process, as suggested by the model described above.

The formation of form X required ATP (Fig. 2b), like the formation and dissociation of D-loops^{3,8,55}. Moreover, when ADP was added to the reaction system after the complete dissociation of D-loops and incubated, the duplex DNA was completely relaxed and no form X DNA was formed during the second incubation (Fig. 3, lane 18). This correlates with our previous observation that after the inactivation of form I DNA by formation and dissociation of D-loops, addition of ADP results in the full reactivation of the duplex⁵⁵.

On the other hand, formation of form X was greatly reduced when homologous single-stranded fragments were omitted or replaced by heterologous ones (Fig. 2b). Moreover, when we compared the initial velocity, the difference between the formation of form X in the presence of homologous single-stranded

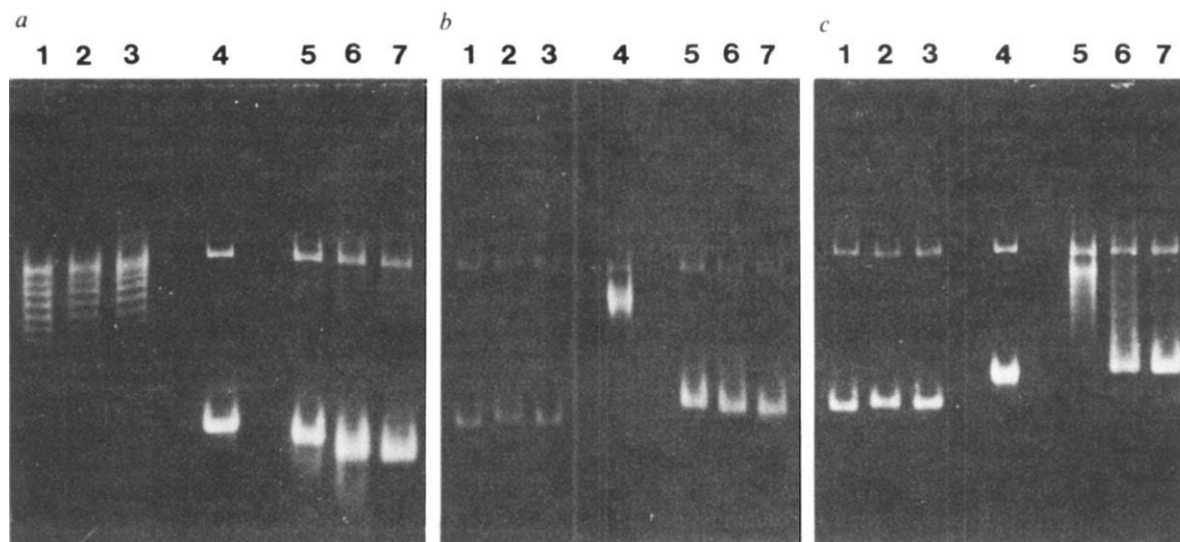


Fig. 4 Effect of ethidium bromide on the migration of the products in gel electrophoresis. Conditions for the reaction and electrophoresis are given in Figs 2 and 3 legends. Samples were electrophoresed in the absence of ethidium bromide (a), in the presence of 0.15 μM ethidium bromide (b) or in the presence of 0.7 μM ethidium bromide (c). For lanes 1–3, both RecA protein and the first incubation were omitted; for lanes 5–7, the first incubation in complete reaction mixture was carried out for 50 min at 37°C. The period of the second incubation with topoisomerase was 20 s (lanes 1 and 5), 5 min (lanes 2 and 6) or 15 min (lanes 3 and 7). Lane 4, untreated form I DNA.

fragments and its formation in the absence of homologous fragments was even greater, the former being 30-fold larger (M.I. and T.S., unpublished observation). However, the formation of form X DNA does occur at a very low rate in the absence of single-stranded DNA and is slightly stimulated by the addition of heterologous single-stranded fragments, but only if double-stranded DNA substrate is form I DNA (Fig. 2b and Fig. 3, lanes 15, 16). When double-stranded DNA substrate is relaxed DNA (form IV DNA), the formation of form X DNA seems to require homologous single-stranded fragments (T.S. M.I. and T.O., unpublished observation, and A. M. Wu, and C. M. Radding, personal communication). These results suggest that the unwinding of the double helix by RecA protein might be initiated at all D-loop sites and also initiated at a very low rate at the transient denatured sites in superhelical DNA³¹⁻³³. The initiation at the latter sites is stimulated by any single-stranded DNA (data not shown) as the unwinding in the presence of ATPγS (ref. 23).

The enzymes which catalyse unwinding of the double helix in the presence of a high-energy cofactor such as ATP are called DNA helicases, and include DNA helicase I, II (refs 34-36) and III (refs 37, 38) from *E. coli*, *E. coli* Rep protein³⁹⁻⁴² and ATP-dependent DNases from *E. coli*⁴³⁻⁴⁵ and *Haemophilus influenzae*⁴⁶. As a DNA helicase, RecA protein is unique; it unwinds closed-circular DNA without strand termini, whereas all other DNA helicases require a single-stranded tail, gap or free end in the DNA to initiate the unwinding of the double helix. Moreover, RecA protein has not been found to initiate unwinding from a single-stranded tail or gap (ref. 47 and R. P. Cunningham, unpublished observation). DNA helicase activity of RecA protein seems to have a role in the elongation of heteroduplex joints by RecA protein *in vitro*¹⁰⁻¹² and in branch migration during recombination *in vivo*¹⁵.

The present study also indicates that closed-circular double-stranded DNA with an extraordinarily large number of negative-superhelical turns can be formed in physiological conditions by the two proteins, RecA protein and topoisomerase I. In eukaryotes in which gyrase activity has not been found, RecA-like protein⁴⁸ and topoisomerase I might promote a high degree of supercoiling.

We thank Dr Jean M. Michalec for reading the manuscript, Drs C. M. Radding and W. K. Holloman for communicating their results before publication and Dr C. M. Radding for stimulating discussions. This study was supported in part by a grant from the Science and Technology Agency, Japan, and by a grant from the Ministry of Education, Science and Culture, Japan.

Received 6 April; accepted 1 July 1982.

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Errata

In the letter 'Curare can open and block ionic channels associated with cholinergic receptors' by A. Trautmann *Nature* **298**, 272-275 (1982), the symbols in Table 1 representing the different types of transitions observed should be rotated through 180°.

In the letter 'Electrical stimulation of hindlimb increases neuronal cell death in chick embryo' by R. W. Oppenheim & R. Nunez *Nature* **295**, 57-59 (1982), in Fig. 2 the bars representing pooled data should be labelled CE & SE (left-hand bar) and CU & SU (right).

Corrigenda

In the letter 'Differences in the kinetics of rod and cone synaptic transmission' by J. L. Schnapf & D. R. Copenhagen, *Nature* **296**, 862-864 (1982), the plots of the equation shown in Fig. 1 legend (that is, in Figs 1c, 2c and 3) do not have large enough undershoots. This in turn leads to an overestimate of the decay time of the impulse response. The impulse response actually falls to 1/e its peak value in 80 ms for the rod synapse and 10 ms for the cone synapse (not 131 ms and 16 ms as stated in the second-to-last paragraph). The major conclusions of the paper remain unchanged: the synapses display high and low pass filter characteristics and the rod synapse is approximately 10 times slower than the cone synapse.

In the letter 'Directed effector cells selectively lyse human tumour cells' by C. B. Simone, *Nature* **297**, 234-236 (1982), line 16 on page 236 states that "direct effector cells... cause no adverse reactions *in vivo* (mice and one human)." Instead of "no adverse reactions", the author should have stated that the patient did have two fevers during the five day treatment which responded to acetoaminophen. However, no other toxicities were noted. Obviously, a full trial needs to be done to explore the efficacy of directed effector cell treatment.

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BOOK REVIEWS

World on a ledger

Timothy O'Riordan

IT IS worth casting one's mind back ten years. Oil prices were about \$2 a barrel; OPEC was in existence but seemingly compliant to Western interests. There had been a number of years of unprecedented economic prosperity in most Western and some Third World countries, though the Soviet bloc was experiencing its perennial economic tribulations. Prospects of widespread affluence and material comfort appeared almost a reality.

The only flies in the ointment, it seemed, were that environmental quality (however defined) was palpably worsening and fears of resource scarcity were rife: yet economists continued to tell their political masters that there need be no resource-scarcity limits to growth. This left the "environmental problem" and that was to be tackled at the United Nations Conference on the Human Environment held in Stockholm in June 1972. This conference was full of fanfare and noble rhetoric. If it did nothing else it showed that environmental protection was as much a political issue as a scientific one, and that poverty and environmental destruction were as much interlinked as affluence and environmental mismanagement.

The Stockholm Conference spawned the UN Environment Programme, a rather sickly fledgling which had to find a niche in "far off" Kenya, and a purpose. Over the past ten years UNEP has suffered setbacks as well as experienced some achievements, but in general its main problem is to convert an unwieldy bureaucracy into really meaningful action. Where it has proved its worth, however, is in the monitoring of global environmental problems and processes (notably with respect to chemical cycles and chemical transfers within these cycles) and in its encouragement of international scientific collaboration over matters of global and regional environmental concern.

This book is a fine testament to those important achievements. Its aim is to produce a scientific audit of how the global environment has altered over the ten years since the Stockholm Conference. To this end UNEP devoted considerable effort to commission reports from a large number of scientists, official and non-governmental bodies, established a Senior Scientific Advisory Board to manage the operation and asked three distinguished scholars to edit this awesome mountain of material into a comprehensive and comprehensible synthesis. A particular feature of this book

The World Environment 1972-1982: A Report by The United Nations Environment Programme. Edited by Martin W. Holdgate, Mohammed Kassas and Gilbert F. White. Pp.637. Hbk ISBN 0-907567-13-4; pbk ISBN 0-907567-14-2. (Tycooly International, Dublin: 1982.) Hbk £50, pbk £25.

lies in its almost encyclopaedic factual approach: graphs, tables and statistics abound. It also concentrates on a backward look. This is not one of the ever more prevalent "think tank scenario productions" with myriads of graphs looking like the coloured tails of an aerobic team. Insofar as it is possible to maintain scientific objectivity, the editors have been successful, and they frequently point out when data are unavailable, difficult to interpret or based on poor experimental evidence.

The book itself is in three parts: the first is concerned with sectors of the physical environment — the atmosphere, marine water, inland water, the lithosphere and its mineral resources, the biosphere and its resources of food, fibre, timber and fuel. Trends in pollution, productivity, human use and other major features are noted, the capacity of the various environmental systems to cope with present demands upon them are also analysed, and both national and international efforts to safeguard these environmental systems are described in some detail. The second part concentrates on human use patterns — on population distribution and growth, on changing patterns of health and disease, and on the conditions of major settlements. The third section covers major human activities affecting environmental systems — industrial developments, the generation and transmission of energy, transportation and tourism.

Collectively the authors are convinced that lack of understanding rather than lack of skills is the one main reason why environmentally sustainable development cannot proceed, and devote an important chapter to recent efforts to upgrade environmental education. They are equally convinced that global survival cannot be guaranteed so long as the present diversion of resources towards military training and increasingly destructive armaments (both conventional and nuclear — though the distinction is now more blurred) continues. So they include a chapter on the ecological and economic consequences of wars

through the 1970s and to the environmental implication of both nuclear and chemical/biological warfare. The authors are not optimistic about the much-longed-for reduction in the arms race with its liberating release of resources and cash for desperately needed environmental improvements. In this chapter, as in the rest, they display an impressive grasp of the key issues and a precise analytical clarity which deserves much praise.

The editors' conclusions are both sobering and hesitant. They confess that, though much has been done over the past ten and more years to improve understanding of the main environmental systems, there are still serious gaps in both scientific understanding and the data base. They note especially the shortage of reliable quantitative information about environmental changes in the developing world. In other respects what data are available can only be described as preliminary (e.g. tropical forest loss and especially the ecological implications of this loss). Similarly detailed estimates of the increase of the desert margins are not yet available, nor are there reliable statistics on the incidence and spread of major diseases. They observe (p.623) that

even an imperfect series of environmental statistics, drawing together what we have (and, through its manifest inadequacies, providing a stimulus to better observation) would be an improvement on the present situation.

A second important conclusion from this study is the essential physical unity of the global ecosystem in terms of biochemical cycles. Each of these interact and hence each is affected by human use and misuse in a most complex manner. But how these interactions work and what will be the consequence if current alterations to these cycles continue simply are not known.

The editors' conclusions are also sobering, because enough is known to cause us all to take the matter very seriously yet the global community of nation states does not appear to want to respond. Ten years after Stockholm the world is in a very different state. Economic recession is almost universal, unemployment is growing almost everywhere and prospects for the citizens of tomorrow, in terms of jobs, wealth and leisure as conventionally defined, look dim indeed. Economic recession has also led to attempts to cut public expenditure. In such an atmosphere international organizations operating

seemingly at the periphery of global economic interests are most vulnerable. Thus UNEP itself is threatened (see *Nature* 3 June pp.348–350). Its budget is declining in real terms and it has failed to co-ordinate the efforts of the various UN agencies, jealous to protect their own sand boxes.

Sadly, this extremely valuable book is symbolic of the failure of UNEP. It is detailed and scientific, steering clear of tackling head on the political necessities for action: it looks backward most of the time but when it peers into the future it only sees uncertainty and ambiguity — two qualities that are readily exploited by politicians living for short term expediency. Finally, the book is an expensive encyclopaedia and as such inaccessible to those lay people who would most want to read it. Those wanting a more accessible document covering much the same material, though in a more political context, should read Erik Eckholm's *Down to Earth* (Pluto Books, 1982). □

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Yeast biography

J.M. Mitchison & P.A. Fantes

The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance. Edited by Jeffrey N. Strathern *et al.* Pp. 751. ISBN 0-87969-139-5. (Cold Spring Harbor Laboratory: 1982.) \$75 (US), \$90 (elsewhere).

FOR at least a century, yeast has been as interesting to the biologist as it has been important to the brewer, the wine maker and the baker. This interest has increased markedly in recent years as it has become clear that yeast can provide a good model for events taking place in other and higher eukaryotic cells. In particular, genetic manipulation has become relatively easy because of the "good" genetics of yeast, the development of hybrid plasmids which will grow both in yeast and *E.coli*, and efficient methods for transformation.

This excellent book is a sign of the times — perhaps only half a sign, since the second, companion volume on metabolism and gene expression is not due until the autumn. The two books will not provide a comprehensive survey of the biology of yeast since they omit a number of topics, including the applied aspects; but they do cover many of the exciting areas of modern yeast research and there is no question of the value of this first volume. The articles are written by experts and will remain definitive statements for at least

several years, even in fast moving fields. They are a good deal more than references hung on a thin thread of text, and many of them include "overviews" which will be especially valuable to the non-specialist.

The longest article is a comprehensive account by Dujon of the dense and complex field of mitochondrial genetics. The shortest is by Roman who gives a succinct historical review of yeast genetics pointing out that it precedes the genetics of *E. coli* by more than ten years. Mortimer and Schild describe the methods of genetic mapping and provide a valuable and up-to-date map of 312 genes on the 17 chromosomes (and fragments) of *S. cerevisiae*. Fogel, Mortimer and Lusnak review the problem of meiotic gene conversion with the delightful sub-title of "Wanderings on a Foreign Stand". Their contribution is for the experts but includes a nice section on the possibility of using chromosomes modified by recombinant DNA techniques to ask direct questions about polarity and conversion. Herskowitz and Oshima give a clear and concise account of the mating type system; this is an outstanding review of the genetics and molecular biology of a fascinating aspect of yeast biology. Esposito and Klapholz deal with meiosis and ascospore development in a good article which includes a number of peripheral topics. Haynes and Kunz review the question of DNA repair and mutagenesis — a complex area which will become increasingly important in the future.

At the slightly higher level of cell physiology and cytology, Byers gives an authoritative account of the ultrastructure of the yeast life cycle where there is a real mystery in the existence of a mitotic spindle without condensed chromosomes. Perhaps this is because of the absence of H1 histone, as Fangman and Zakian suggest in their interesting review of genome structure and replication. Thorner describes the intriguing pheromones of budding yeast; these regulatory proteins "phase" cells of the opposite mating type. There is an article of high quality on the cell cycle by Pringle and Hartwell, who analyse the results from *cdc* mutants and come to a conclusion about cell cycle controls which is more complex than earlier ones. The "circuitry" of the cell cycle has proliferated into a series of parallel pathways and some of the elegant simplicity of the earlier models has been lost — but this is a criticism of life rather than of the authors.

Although the title firmly restricts the book to *Saccharomyces*, it is a little sad that there are not more comparisons both with other yeasts and with other eukaryotic cells. This, however, is a minor point. The major one is that this important book should be available to all who work with yeast and related cells. □

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Monocot characters

Peter D. Moore

The Monocotyledons: A Comparative Study. By Rolf M.T. Dahlgren and H. Trevor Clifford. Pp.378. ISBN 0-12-200680-1. (Academic: 1982.) £48, \$98.50.

A COMPREHENSIVE survey of the taxonomic and phylogenetic classification of the Monocotyledones has long been needed. Perhaps it is the sheer size of the task which has deterred prospective authors, together with the wide range of techniques and evidence which is now available to those who wish to investigate evolutionary relationships.

Happily, this task has now been undertaken and it has been tackled in an ambitious and refreshingly novel manner. The bulk of this well-illustrated book is taken up with a "survey of the distribution of selected characters and their states" within the monocotyledons. Here the authors begin with vegetative morphological characters (such as bulbs, petioles, leaf venation), progress through reproductive features (floral structures, pollen morphology, fruit types) and then consider biogeographical and physiological characteristics (salt tolerance, cyanogenesis, secondary compounds and so on). Within each of these sections the occurrence and variation of that feature within the monocots is considered and often its distribution in the group is illustrated using ordination-type diagrams of floral orders.

Finally, the classification of the monocots is considered in the light of these character analyses. The proposal of the authors is that 26 orders should be recognized, of which 11 contain just one family and a further 7 two or three families only. This may be regarded as the outcome of undue splitting, but three alternative systems are presented which permit higher levels of fusions, at the extreme recognizing only 16 orders. Also, relationships with allied dicot groups are kept firmly in mind.

This is a novel work which has broken with traditional taxonomic treatments by approaching the study of the monocots via characters rather than by dealing with proposed orders in a systematic fashion. This will prove particularly valuable to those who may have interests in specific morphological or physiological traits, from ovule types to saponins, or even in such topics as fungal or insect host-specificity. Not only does this arrangement make it possible to extract information simply and rapidly, but it also provides a rational and objective approach to the final synthesis, giving equal weight, as it does, to all of the varied lines of evidence which are now available to assist in the classification of the monocotyledons. □

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Interpreting the rocks of deepest Africa

H.B.S. Cooke

Crustal Evolution of Southern Africa: 3.8 Billion Years of Earth History. By A.J. Tankard *et al.* Pp.524. ISBN 0-387-90608-8/3-540-90608-8. (Springer-Verlag: 1982.) DM118, \$55.

THE systematic geological study of South Africa began before the middle of the nineteenth century and the first map of the Cape Colony by Andrew Geddes Bain appeared in 1852, by which time he had already worked out the essential Phanerozoic stratigraphy. Mineral exploitation subsequently showed the importance of the Precambrian and much attention has been devoted to unravelling the tangled skein of "basement" geology. It is only in the past decade or so that emphasis has shifted from a largely descriptive and stratigraphic attack to more detailed studies concerned with provenance and environmental interpretation. Tankard and his collaborators have produced a volume in keeping with this modern view, one that is radically different from the traditional text on regional geology; their approach is highly interpretative, strongly process-orientated and stresses the dynamic evolution of the crust. The work is dedicated to the great South African geologist and pioneer protagonist of continental drift, Alex L. du Toit, and he would certainly have enjoyed it.

The area covered is essentially the tip of the African continent south of about 16°S, embracing the Republic of South Africa and the adjoining territories of Namibia, Botswana, Lesotho, Swaziland and Zimbabwe. The introductory chapter outlines the basic tectonic framework and delimits the boundaries of major structural "provinces" that underlie the relatively undeformed cover sequences, some of the latter being as old as Late Archean. Each province is a geographical region that shares a number of geological parameters — notably the predominant radiometric age and metamorphic style — the sum of which differentiate the region from the adjoining ones. Thus the provinces are not "cratons" separated by "mobile belts", for the mobile belts of one age commonly become the cratons of a later stage. The Sand River Gneisses in the Limpopo Province south-east of Messina have been dated at 3.8 Ga and are some of the oldest rocks known on Earth; hence the subtitle of the volume. Thus the chronology of the region is a particularly long one.

For the subdivision of the Precambrian, the authors have chosen to use the standard international divisions, Archean and Proterozoic (with Early, Middle and Late subdivisions) rather than employ the regional units adopted by the South African Committee for Stratigraphy (SACS — see Handbook 8 of the Geological Survey of South Africa, 1980).

Although this may be palatable to the international readership, it is a little unfortunate as the SACS units were selected because they are "natural" local divisions and, in point of fact, correspond well with the stages into which the authors divide the evolutionary history of the South African crust. Thus their Stage 1, "Archean Crustal Evolution", does not cover the Late Archean but does correspond to the Swazian, while Stage 2 is labelled "Early Proterozoic Crustal Development" but includes the Late Archean; it corresponds to Randian plus Vaalian. In some other respects, however, the authors use of supergroup where the SACS scheme employs "sequence" (e.g. for the Karoo) is decidedly an improvement.

The text is divided into sections, each of which is devoted to one of the five major stages. Within each section the treatment is generally similar with chapters that deal primarily with a characteristic terrain and a particular province. For example, Chapter 2 is entitled "Granite-Greenstone Terrane: Kaapvaal Province" and incorporates discussion of the now classic Swaziland supergroup, although it does also include some consideration of the Zimbabwe province.

Throughout the volume there is a tendency to provide more petrologic and geochemical data for igneous and metamorphic suites than there is descriptive material for sedimentary sequences, for which only a minimum of stratigraphic or lithostratigraphic data are provided and emphasis is strongly on depositional environments and basinal analysis. The text is thus unusual in its stress on processes and causes, employing the interrelationships between observed structures to unravel the succession of events responsible for the state of the rocks that we now see. On the whole a good balance has been maintained, although the Phanerozoic may be under-played, in marked contrast to the more conventional over-emphasis on this span of geological time.

The illustrations are clear and helpful, although the quality of photographic reproduction leaves something to be desired. Literature coverage is extensive and the authors are to be congratulated on the high quality and up-to-date nature of the synthesis. Not everyone will agree with all of the conclusions and alternative interpretations are not always considered, but this is inevitable with a work of this scope and one must admire the coherence of the whole presentation. It is not easy reading and it is inevitable that many stratigraphic units are mentioned without an accompanying description, so a companion stratigraphic text (such as the recent SACS volume) would be useful for reference.

The book was written, as the preface states, "for advanced undergraduates, graduate students, and professional geologists worldwide". For all of them it should provide a new perspective and an essential tool. □

H.B.S. Cooke was until recently Carnegie Professor of Geology at Dalhousie University, and is now a geological consultant in British Columbia. He was for many years on the staff of the University of Witwatersrand, Johannesburg.

The little bang

R.G. Evans

Inertial Confinement Fusion. By James J. Duderstadt and Gregory A. Moses. Pp.347. ISBN 0-471-09050-6. (Wiley: 1982.) £36, \$64.50.

ALTHOUGH the intensive research effort now being devoted to controlled thermonuclear fusion may appear to be a consequence of the current emphasis on energy projects, the basic ideas date back to the early 1950s. The main approach to the problem of heating and confining a plasma at a temperature of one hundred million degrees has been to use a large and suitably shaped magnetic field. These magnetic confinement machines, for example the JET machine now being built at Culham, are relatively close to the conditions needed for net energy production. But there are still problems with the toroidal geometry for a reactor system, particularly regarding maintenance, and the breeding of tritium to replace that used up in the thermonuclear reactions.

An alternative approach to fusion is not to confine the plasma at all but to utilize the reactions that occur before the plasma pressure overcomes its inertia and it blows apart, hence the slight misnomer of inertial confinement fusion (ICF). At one extreme this technique has been proven with devastating efficiency in the thermonuclear or hydrogen bomb. To reduce the explosive yield of a hydrogen bomb to manageable proportions requires the rapid compression of a small pellet of deuterium/tritium fuel to densities of a few hundred grams per cubic centimetre. The technical means to do this were not available until the advent of high-power lasers in the late 1960s and the major declassification of inertial confinement fusion occurred in 1972. There was early optimism for "break even" experiments with fairly modest lasers and this spurred on laser development programmes in the United States, the Soviet Union and to a lesser extent Japan and Europe. The promise of the early theoretical predictions has been somewhat tempered in the light of experimental testing, however; the current

laser fusion projects are as expensive as the large magnetic machines.

Despite much activity in this field, it has taken ten years for the first book to appear dealing with the whole range of physics involved. *Inertial Confinement Fusion* gives a commendably comprehensive coverage of the subject dealing for instance with the thermonuclear reactions themselves, plasma physics, hydrodynamics, lasers, particle beams and reactor problems. The book is very thorough on the different driver systems and describes high power lasers, relativistic electron beams, and both light and heavy ion drivers. In dealing with such diverse topics the book will inevitably leave many readers dissatisfied with particular details. For instance the section on laser plasma interaction fails to deal adequately with the important areas of stimulated Raman and Brillouin scattering, and the problem of symmetry of compression is barely touched upon. It would also have been invaluable to have examined the reasons for the failure of laser fusion to fulfil its early promise.

Several factual errors have crept in through an apparent zeal for making the information as up to date as possible; some ICF facilities are declared to be in existence when they have yet to obtain full financial approval. The book is generally very readable, however, and has its lighter moments — for instance the definition of “anomalous” as meaning inadequately understood, and the reference to “the thrilling days of yesteryear” in relation to heavy ion fusion where the high cost of experimental hardware has so far shielded the theory and computational predictions from the rigours of reality.

Aside from these criticisms the book fills a large gap in providing graduate research workers with an up-to-date description of a wide ranging field. There is a large number of references, although these are mostly to work in laboratories in the United States, and perhaps too large a fraction are to internal laboratory reports which might not be widely available.

“The power of the stars” may not yet be with us, but at least Duderstadt and Moses have confronted us with most (though not all) of the problems of the inertial confinement approach to fusion. □

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Archaeoastronomy

Papers presented at the archaeoastronomy symposium held in September last year at Queen's College, Oxford (reported by Aubrey Burl in *Nature* 293, 335; 1981) have been published in book form by Cambridge University Press. The proceedings have been divided between two companion volumes, *Archaeoastronomy in the New World* (edited by A.F. Aveni) and *Archaeoastronomy in the Old World* (edited by D.C. Heggie). Prices are £16, \$29.95 and £20, \$37.50 respectively.

Gell and Coombs for the nineteen eighties

Fred S. Rosen

Clinical Aspects of Immunology, 4th Edn. Two volumes, pp.1,751. Edited by P.J. Lachmann and D.K. Peters. ISBN 0-632-00702-8. (Blackwell Scientific: 1982.) £90, \$195.

IMMUNOLOGY started as a clinical science two centuries ago in rural Gloucestershire. A hundred years later Pasteur gave the subject renewed impetus by his studies in chickens. In recent decades the greatest progress in illuminating the arcana of the immune response has come from the study of inbred strains of mice, and the clinical aspect of immunology has almost become a quiet backwater of the subject. So this revised and expanded edition of “Gell and Coombs” classic work is welcome addition to the slim space on library shelves devoted to the clinical aspects of immunology.

The first volume consists of 744 pages of basic immunology and the second of 1,006 pages of the clinically relevant applications of immunology to disease entities. Because of the nature of the subject matter the first volume is the more cohesive and satisfying of the two. It starts with six succinct chapters on the relevant globulins and cells of the immune response. The illustrations and diagrams are copious and germane to the text and each chapter ends with a useful list of references, features which remain consistently good throughout the work.

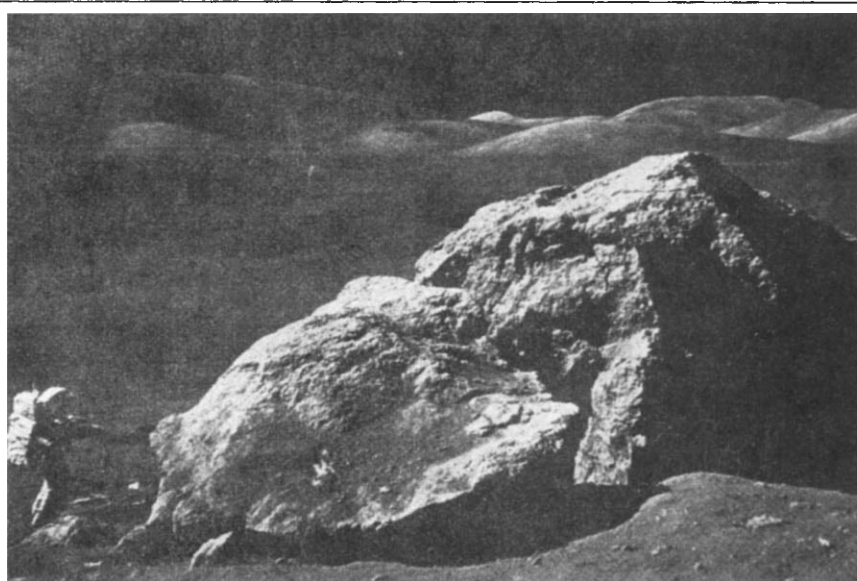
The second section in Vol. 1 is devoted to a discussion of cellular interactions and contains a unique contribution from J. H. Humphrey on the fate of antigens. Two further sections are concerned with technology that is clinically relevant, such as radioimmunoassays, rosette-forming

reactions, detection of immune complexes, tissue typing and monoclonal antibodies, and with the limited approaches to suppressing or stimulating the immune response. The final section of the first volume ends with a presentation of the pathophysiology of allergy and immune injury. Special praise is due T.A.E. Platts-Mills for his comprehensive, if not exhaustive, presentation of immediate hypersensitivity. This contribution is a mini-textbook on its own and the best current presentation of the subject matter that I know of.

The first volume is a hard act to follow, but although Vol. 2 is more uneven it in the main sustains the high quality of its companion. It deals with organ-specific immunologically-mediated disease, rheumatology, transplantation and infection. Although the text is purported to be written in English, jargon such as “. . . patients with NPC have high titres of anti-D, and certain patients with BL have high levels of anti-R” leave the reader groping to decipher the meaning.

This text, like all scientific and medical works these days, is expensive but it is well worth the cost to all those who practise clinical immunology. It is more current and broader in its coverage than the two other multi-volume competitors in the field. In an arena of rapidly expanding information, Lachmann and Peters have done a superb job of comprehensively assembling the diverse subject matter that constitutes the clinical aspects of immunology. □

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Harrison Schmitt in the valley of Taurus-Littrow during the Apollo 17 mission to the Moon. During their 22 hours of exploration Schmitt and Eugene A. Cernan investigated the major geological formations in the valley and collected 110 kg of rock and soil samples, including a 3m core — material which among other things provided new data on the early crustal history of the Moon. The picture is taken from *The Solar System and Its Strange Objects*, edited by Brian J. Skinner, a new addition to the series *Earth and Its Inhabitants: Readings from American Scientist*. The book is published by William Kaufmann, price £8.40, \$11.95 in paperback.

NASA photograph AS17-140-21496